INTERACTION OF ALCOHOL AND SMOKING IN THE PATHOGENESIS OF UPPER DIGESTIVE TRACT CANCERS – POSSIBLE CHEMOPREVENTION WITH CYSTEINE

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ACADEMIC DISSERTATION

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CONTENTS

ABBREVIATIONS

ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
AUC	area under the curve
CFU	colony forming units
CYP	cytochrome P450
DNA	deoxyribonucleic acid
G	guanine
GI	gastrointestinal
IARC	International Agency for Research on Cancer
Km	Michaelis constant
MDA	methyl-djenkolic acid
MEOS	microsomal ethanol oxidizing system
MTCA	methyl-thiazolidine-carboxylic acid
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NMTCA	nitrosothiazolidine 4-carboxylic acid
OR	odds ratio
PAH	polycyclic aromatic hydrocarbons
PCA	perchloric acid
RR	relative risk
SEM	standard error of the mean
Ssp	subspecies
T	thymine

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals:

I Salaspuro V, Nyfors S, Heine R, Siitonen A, Salaspuro M, Jousimies-Somer H (1999) Ethanol oxidation and acetaldehyde production in vitro by human intestinal strains of *Escherichia coli* under aerobic, microaerobic, and anaerobic conditions. Scand J Gastronterol 34:967-973.

II Salaspuro V, Salaspuro M (2004) Synergistic effect of alcohol drinking and smoking on in vivo acetaldehyde concentration in saliva. Int J Cancer 111:480-483.

III Salaspuro V, Hietala J, Kaihovaara P, Pihlajarinne L, Marvola M, Salaspuro M (2002) Removal of acetaldehyde from saliva by a slow-release buccal tablet of L-cysteine. Int J Cancer 97:361-364.

IV Salaspuro V, Hietala J, Marvola M, Salaspuro M (2006) Eliminating carcinogenic acetaldehyde by cysteine from saliva during smoking. Cancer Epidemiol Biomarkers Prev 15:146-9.

ABSTRACT

BACKGROUND

Alcohol consumption and smoking are undisputedly the main risk factors for upper digestive tract cancers in humans. Billions of people worldwide engage in these behaviours, thus creating disease burden. Strong experimental and human genetic linkage data suggest that acetaldehyde is one of the major factors behind the carcinogenic effect of alcohol drinking. In the digestive tract, acetaldehyde is mainly formed by microbial metabolism of ethanol.

Acetaldehyde is also a major constituent of tobacco smoke. Thus, acetaldehyde from both of these sources may have an interacting carcinogenic effect in the human upper digestive tract. Earlier experimental studies have shown that the amino acid cysteine has the ability to bind reactive acetaldehyde. The local use of cysteine might therefore be able to prevent the harmful effects of acetaldehyde.

AIMS

The first aim of this thesis was to elucidate the capacity of microbes to produce acetaldehyde from ethanol in different atmospheric conditions prevailing in the human digestive tract.

The second aim was to investigate acetaldehyde production and exposure in the human mouth resulting from alcohol ingestion and tobacco smoking *in vivo*. Thirdly, specific L-cysteine products were prepared to examine their efficacy in the binding of salivary acetaldehyde in order to reduce the exposure of the upper digestive tract to acetaldehyde.

METHODS

Firstly, the acetaldehyde production and ethanol fermentation of *Escherichia coli* – an important member of the human microbiota – was measured *in vitro* in anaerobic, aerobic and microaerobic conditions.

In other studies, acetaldehyde was measured in the saliva of human volunteers *in vivo* during alcohol metabolism, during tobacco smoking and during the combined use of alcohol and tobacco. The ability of L-cysteine to eliminate acetaldehyde during alcohol metabolism was also measured by using a specifically developed slow releasing buccal tablet. In addition, the ability of L-cysteine to eliminate acetaldehyde dissolved in saliva from tobacco smoke was examined by using a L-cysteine-containing tablet that was sucked during smoking.

RESULTS AND CONCLUSIONS

In vitro results with *E. coli* demonstrate that ADH activity-possessing bacteria representing the normal human microbiota can be one regulatory factor in acetaldehyde production, leading to marked formation of carcinogenic acetaldehyde from ethanol, especially under aerobic or microaerobic conditions.

Acetaldehyde produced in the oral cavity during ethanol challenge was significantly decreased by a buccal L-cysteine -releasing tablet. L-cysteine efficiently bound reactive acetaldehyde by forming a stable thiazolidine compound, thus preventing the reactivity of acetaldehyde against mucosal cells. Furthermore,

smokers were established to have significantly increased acetaldehyde exposure during ethanol metabolism even when not smoking. However, acetaldehyde exposure is dramatically further increased during active tobacco smoking. Thus, the elevated aerodigestive tract cancer risk observed in smokers may be the result of the acetaldehyde exposure from tobacco smoke. Moreover, the dramatically increased exposure to carcinogenic acetaldehyde caused by simultaneous smoking and drinking may explain the synergistic and multiplicative effect of alcohol consumption and tobacco smoking on upper digestive tract carcinogenesis.

The last study of this thesis shows that smoking-derived acetaldehyde can be totally removed by using a tablet containing L-cysteine. Hence, a cysteine tablet sucked during each smoking period could be used to eliminate acetaldehyde exposure, thus potentially preventing upper digestive tract cancers in smokers.

INTRODUCTION

A large proportion of upper digestive tract cancers are attributable to alcohol consumption and smoking. These risk factors account for over 75% of all cases in developed countries (Franceschi et al., 1990; La Vecchia et al., 2004). Moreover, evidence indicates that the combined use of alcohol and tobacco increases the risk of these cancers multiplicatively, i.e. each factor multiplies the effect of the other. Compared with never-smokers and alcohol abstainers, the relative risk of upper digestive tract cancers may be more than 100-fold higher in heavy smokers and heavy drinkers. Already the consumption of 20 - 30 g (two drinks) of alcohol per day markedly increases the risk of upper digestive tract cancer (Bagnardi et al., 2001b). Thus, the consumption levels and trends of use of alcohol and tobacco have a major influence on the total incidence and mortality rates of oral, pharyngeal, laryngeal and oesophageal cancers.

Many possible mechanisms by which alcohol causes cancer exist. However, cell culture and animal studies strongly suggest that acetaldehyde is the main factor responsible for the carcinogenic effect of alcohol, whereas ethanol *per se* is not a carcinogen (IARC, 1988; IARC, 1999). Very recent molecular studies have shown that acetaldehyde induces carcinogenic changes in concentrations that have been measured in human saliva after a moderate dose of alcohol (Brooks and Theruvathu, 2005).

In addition to experimental studies, polymorphism of ethanol-, and acetaldehyde- metabolizing genes – alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) – can be used to assess the association between acetaldehyde and cancer in humans. The risk of upper digestive tract cancers is markedly increased in individuals with impaired acetaldehyde removal due to a mutation in the ALDH2 gene (Yokoyama et al., 1998). The mutation, which is common in Asians, results in elevated levels of acetaldehyde in saliva after alcohol challenge, thus subjecting these individuals to higher local acetaldehyde exposure (Väkeväinen et al., 2000) and greater risk of upper digestive tract cancers than individuals with a normal ALDH2 enzyme. Caucasian heavy drinkers and alcoholics with high-activity ADH and enhanced local acetaldehyde production in the mouth presumably also have an increased risk of upper aerodigestive tract cancers (Homann et al., 2005; Visapää et al., 2004).

Acetaldehyde is known to be locally formed in the upper digestive tract, mainly by microbes representing normal oral flora (Jokelainen et al., 1996a). Salivary glands and mucosal cells may also act as minor contributors to the acetaldehyde concentration present in saliva during ethanol metabolism (Visapää et al., 2002; Väkeväinen et al., 2000). Microbial acetaldehyde production is strongly influenced by individual factors and differences in oral flora (Homann et al., 2000). Thus, elucidation of genetic and environmental factors contributing to the

local concentration of carcinogenic acetaldehyde in saliva is essential when considering individual risk and possible prevention of upper digestive tract cancers.

Acetaldehyde is also one of the major components of tobacco smoke (Hoffmann and Hoffmann, 1997). Although tobacco smoke contains several substances classified as carcinogens, their individual roles *in vivo* remain obscure. Thus, elucidation of tobacco-derived acetaldehyde as a possible factor underlying aerodigestive tract cancers is also needed. The pathogenesis behind the multiplicative carcinogenic potential associated with the combined use of alcohol and tobacco has thus far been unknown. Because acetaldehyde has multiple cancer-promoting mechanisms, this thesis presents a new hypothesis that the combined exposure to acetaldehyde derived from tobacco smoke and microbial alcohol metabolism explains the multiplicative risk effect.

Thiols such as L-cysteine, have been known for decades to bind acetaldehyde. By eliminating toxic acetaldehyde, L-cysteine may protect experimental animals from its lethal effects (Sprince et al., 1975). However, numerous studies have to date failed to show any chemopreventive effect of L-cysteine *in vivo* (Meister, 1989). As acetaldehyde is formed in saliva during alcohol metabolism and also dissolves in saliva during tobacco smoking, the second goal of this thesis was to find a novel method – by using L-cysteine – to reduce the acetaldehyde concentration in saliva and thereby also the local exposure of the upper digestive tract mucosa to carcinogenic acetaldehyde.

REVIEW OF THE LITERATURE

A systematic review was undertaken of all published data on deleterious impact of alcohol consumption on the human upper digestive tract. Literature about the combined effects of alcohol and tobacco smoking was also systematically reviewed. This was followed by a literature review of the data concerning acetaldehyde as a carcinogenic factor related to alcohol consumption and smoking. Finally, different regulatory factors influencing local *in vivo* acetaldehyde concentration in the upper digestive tract were analysed.

Searches were limited to MEDLINE (Ovid medline (R)) from 1966 to 2005 and the English language, and recently updated to include possible new studies on the topic. A PubMed search was also conducted when indicated. These searches were complemented with manual searches of references in other published articles when necessary. Search terms (mapped to Medical Subject Headings of the database) used in combination are defined at the beginning of each section and the number of total identified publications [number in parentheses] is also given. In some cases, the search term was limited to certain subheadings to restrict the focus of the search [indicated in parentheses].

*, indicates that the *search was run for the selected term and only those results were retrieved in which that term represents the central concept of the records.*

Exp, indicates that the *search was run for the selected term in combination with all of its conceptually narrower terms from the tree display.*

1 EPIDEMIOLOGY OF UPPER DIGESTIVE TRACT CANCERS

1.1 Alcohol and upper digestive tract cancers

Search terms:
*Alcohol drinking AND neoplasms (aetiology, epidemiology) [101]
OR
*Alcohol drinking AND *exp* mouth neoplasms (aetiology, epidemiology) [92]
OR
*Alcohol drinking AND *exp* pharyngeal neoplasms (aetiology, epidemiology) [95]
OR
*Alcohol drinking AND laryngeal neoplasms (aetiology, epidemiology) [75]
OR
*Alcohol drinking AND oesophageal neoplasms (aetiology, epidemiology) [109]
OR
*Alcohol drinking AND stomach neoplasms (aetiology, epidemiology) [40]

To cover all meta-analyses, a PubMed search was also performed with the terms *meta-analysis AND cancer AND alcohol drinking* [50].

As a result, 381 publications were reviewed by abstract and were included in the review if the following criteria were met: meta-analysis, cohort or case-control study published as an original article; findings expressed as odds ratios (OR) or relative risks (RR).
Altogether 141 publications met the inclusion criteria; 118 were case-control studies, 16 cohort studies and seven meta-analyses (one was a double publication; furthermore, four additional meta-analyses were found but RRs or ORs were not expressed). Seven of the 141 studies evaluated the association between alcohol consumption and precancerous lesions (e.g. leukoplakia, erytroplakia) instead of cancer. The first meta-analysis (Holman et al., 1996) (16 cohorts and 1 084 733 subjects) reviews the data published in Medline from 1980 to 1994 and covers oropharyngeal, laryngeal and oesophageal but not oral cancer risks. The two most recent meta-analyses review the cancer data to the year 2000 (Bagnardi et al., 2001b) and 2001 (Zeka et al., 2003). In Bagnardi's meta-analysis (235 studies and 117 471 subjects), epidemiological literature on the association between alcohol consumption and the risk of 18 neoplasms, including oral cavity, pharyngeal, laryngeal, oesophageal and stomach cancers was evaluated. In Zeka's meta-analysis (15 studies and 9 508 subjects), epidemiological evidence on associations between alcohol and tobacco consumption and cancers of the oropharynx, pharynx, larynx and oesophagus was reviewed. Two of the seven meta-analyses explored the relationship of alcohol consumption with several alcohol-related conditions (Corrao et al., 1999; Rehm et al., 2003). Corrao's meta-analysis (200 studies and 97 351 subjects) covers malignant neoplasms of the lip, oral cavity and pharynx combined, in addition to oesophageal neoplasms. Rehm's publication (a review of nine meta-analyses) comprises combined oral and oropharyngeal cancers and oesophageal cancer. The very latest meta-analysis by Altieri (20 studies and 42 822 subjects) covers only the association of alcohol consumption and risk of laryngeal cancer based on studies published after 1988

(Altieri et al., 2005). The seventh meta-analysis, covering alcohol consumption and cancers of the digestive tract and larynx, was manually found from references (Doll et al., 1999) (18 cohort studies with over 695 000 subjects and 37 case-control studies). To avoid an enormous number of references, this systematic literature review includes only these meta-analyses complemented with more recent studies after 2001. Thus, 17 case-control studies and six meta-analyses were included. Four of these 17 case-control studies were based on the same study population.

1.1.1 Mouth and pharynx

Strong epidemiological evidence indicates that alcohol consumption increases the risk of cancers of oral cavity and pharynx. The risks are by and large due to total ethanol intake, increasing with the amount of ethanol consumed. All studies confirmed the carcinogenic effect of ethanol on oral and pharyngeal cancers. For oral and pharyngeal cancers (and also for other upper digestive tract cancers), significantly increased risks have been found for fairly quite low doses of ingested alcohol, i.e. 25 g/day which corresponds to approximately two drinks per day. Daily consumption of 0-40 g of ethanol (or 0-4 drinks) increases the relative risk (RR) for oropharyngeal cancer to 1.5-2.06 (Bagnardi et al., 2001b; Zeka et al., 2003; Znaor et al., 2003). The RR for oral cavity and pharyngeal cancers increases with the amount of alcohol consumed. The RR for oral or pharyngeal cancer with daily intake of 50 g and 100 g of alcohol is 2.85 and 6.01, respectively (Bagnardi et al., 2001b). According to Corrao's meta-analysis, the RR for malignancy of the lip, oral cavity and pharynx reaches 10.7 for a daily dose of 100 g of ethanol (Corrao et al., 1999). Moreover, four or more drinks daily results in 7.2-fold RR for oropharyngeal cancer (Zeka et al., 2003). These conclusions are confirmed by the third meta-analysis by Holman et al., in which the RR for oropharyngeal cancer was 5.39 for persons ingesting five or more drinks per day (Holman et al., 1996).

Thus, there is a unanimous, independent causal effect of alcohol on oral and pharyngeal cancers. However, some debate exists about the threshold level. The threshold may vary from 20 to <50 g of ethanol per day for cancers of the pharynx and larynx (Polesel et al., 2005). Furthermore, data are insufficient to conclude that the carcinogenic effect is dependent on a specific type of alcoholic beverage. More important appears to be the total alcohol consumption. The most frequently consumed beverage in each region appears to be the one with the highest association (Bagnardi et al., 2001b).

1.1.2 Larynx

Strong evidence suggests that alcohol drinking is causally related also to laryngeal cancer. The RR seems however, to be somewhat lower than that for oropharyngeal cancer. There is some inconsistency in the cancer risks based on the anatomical site of larynx, which may be due to a classification bias. Tobacco smoking may have a more striking role in laryngeal cancers than alcohol consumption, since results from a case-control study in non-smoking drinkers (40 cases/160 controls) and non-drinking tobacco smokers (68 cases/161 controls) showed no increased risk for laryngeal

cancer in moderate drinkers. However, the risk was increased with elevated alcohol consumption (Bosetti et al., 2002). An estimated 90% of laryngeal cancers are attributable to tobacco, while alcohol explains approximately 58% of cases (Altieri et al., 2002). Nevertheless, the meta-analysis of laryngeal cancer clearly shows that alcohol consumption is strongly associated with an increased risk of laryngeal cancer. The RR for the highest levels of alcohol consumption ranged between 2 and 10, and were 1.07-1.83 for 0-25 g/day, 1.94-4.5 for 50 g/day and 3.95-4.93 for 100 g/day. Some consumption figures are rounded since alcohol consumption levels in different studies are expressed either as drinks/day or g/day. In general, one drink is estimated to contain 10 g of pure ethanol (Altieri et al., 2005; Bagnardi et al., 2001b; Holman et al., 1996; Zeka et al., 2003).

The cancer risk may also differ by laryngeal anatomical subsites, the supraglottis being more closely related to alcohol consumption than the glottis/subglottis (De Stefani et al., 2004). This is logical as the glottis and subglottis are more exposed to inhaled agents. Therefore the cancer-related RR for supraglottic cancers (OR 3.9 95% CI 2.3-6.7) is higher than that of glottic carcinomas (OR 2.1, 95% CI 1.2-3.7) (481 cases) (De Stefani et al., 2004). There is no indication that the carcinogenic effect is dependent on the type of alcoholic beverage (Altieri et al., 2005).

1.1.3 Oesophagus

There is a strong, direct risk for cancer of the oesophagus related to alcohol consumption. The RR for oesophageal cancer associated with alcohol intake are similar in all reviewed meta-analyses. Moreover, a clear dose-response relationship exists with alcohol consumption. According to meta-analyses, for the lowest level of alcohol consumption, i.e. 0-25…40 g of ethanol daily, the RR for oesophageal cancer is between 1.4 and 2.2 (Corrao et al., 1999; Zeka et al., 2003). For the highest level of alcohol consumption, i.e. 100 g/day, the mean RR for oesophageal cancer varies from 4.23 to 10.7 (Bagnardi et al., 2001b; Corrao et al., 1999). For oesophageal cancer risk, the effect of gender in modifying the risk effect has been reported. Women have a slightly but significantly higher risk for oesophageal cancer than men (Bagnardi et al., 2001b).

According to case-control studies, the ORs for oesophageal cancer for the highest alcohol consumption level (>50 ml/day and >40 g/day) are 3.6 in India (Znaor et al., 2003) (566 cases/1711 controls) and 19.5 in Taiwan (Lee et al., 2005a) (531 cases/818 controls). This large difference can perhaps be explained by genetic differences in alcohol and acetaldehyde metabolism related to ethnicity. Large differences are also present in the incidence of oesophageal cancer in Europe. This may be due to the types of alcoholic beverages consumed. The highest incidence rate of oesophageal cancer has been reported in Calvados, France (26.5/100 000 vs. 3-12/100 000 in other areas in the Europe). The specific aetiological cause in this case has been speculated to be the calvados-spirit (Boeing and EPIC Working Group on Dietary Patterns., 2002).

1.1.4 Stomach

Only one meta-analysis reports the RR for alcohol-related stomach cancer (Bagnardi et al., 2001b). According to this, a small but significant and steadily rising stomach cancer risk is present with increasing alcohol consumption, the RR being 1.05, 1.15 and 1.32 for 25 g, 50 g and 100 g of daily alcohol, respectively. On the other hand, Doll's meta-analysis including 18 cohort and 37 case-control studies concluded that there is insufficient evidence that alcohol is involved in the aetiology of stomach cancer (Doll et al., 1999). The majority of reviewed case-control studies concerning stomach cancer have found no statistically significant association between alcohol consumption and gastric cancer (Barstad et al., 2005; Engel et al., 2003; Hamada et al., 2002; Kelley and Duggan, 2003; Key et al., 2004; Lindblad et al., 2005; Nishimoto et al., 2002; Sasazuki et al., 2002), although one study suggested a positive but not significant trend for cancer of the gastric cardia (Rao et al., 2002). The RR for gastric cardia cancer for those who consumed alcohol >322.5 g alcohol/week was 3.0 (range 0.8-11.1). A significant J- or U-shaped effect of drinking on the risk for stomach cancer was found in a Japanese study (787 cases/1007 controls) in which adjustment was made for the *Helicobacter pylori* status (Kikuchi et al., 2002).

This discrepancy in results with regard to stomach cancer and alcohol consumption is also noted in the IARC summary (not included in this review because it was from year 1988) which evaluated 13 cohort studies and 12 case-control studies (IARC, 1988). It concluded that little aggregate data exist to suggest a causal role for drinking of alcoholic beverages in stomach cancer. Furthermore, if alcohol has an aetiological role, it is minor and unproven.

Epidemiological studies show that incidence of gastric cancer has declined sharply in previous decades. On the other hand, in developed countries there has been a rapid increase in the incidence of gastric cancer localized to the cardia (Kelley and Duggan, 2003). The rapid increase in the incidence of oesophageal and gastric carcinomas has been suggested to result from increases in the prevalence of several modifiable risk factors, including alcohol consumption, diet, overweight, gastrooesophageal reflux and definitely tobacco smoking. The well-recognized risk factor for *H. pylori* for stomach cancers would not explain this phenomenon since it is probably a minor cause in developed countries and mainly a risk factor for non-cardia gastric cancer (Engel et al., 2003).

In conclusion, there is an undisputed, level A grade of evidence (from large systematic reviews, meta-analyses and cohort studies) of a positive association between alcohol consumption and cancers of the mouth, pharynx, larynx and oesophagus. Furthermore, this is a dose-response relationship. In the case of stomach cancer, the data are inconsistent and further epidemiological studies with higher alcohol consumption levels are warranted. Thus, this review confirms by and large the conclusions of the systematic review of the IARC from 1988. However, possible biases must be taken into account, which may explain certain discrepancies. Factors such as incidence of cancer, food intake, lifestyle and ethnicity (with regard to different genetic differences in ethanol and/or acetaldehyde metabolism) of the study

population might influence results, especially those related to oesophageal and stomach cancer risk and alcohol consumption. The dose-response effect of alcohol on upper digestive tract cancers without specific adjustments for ethnicity or genetic differences is summarized in Figure 1.

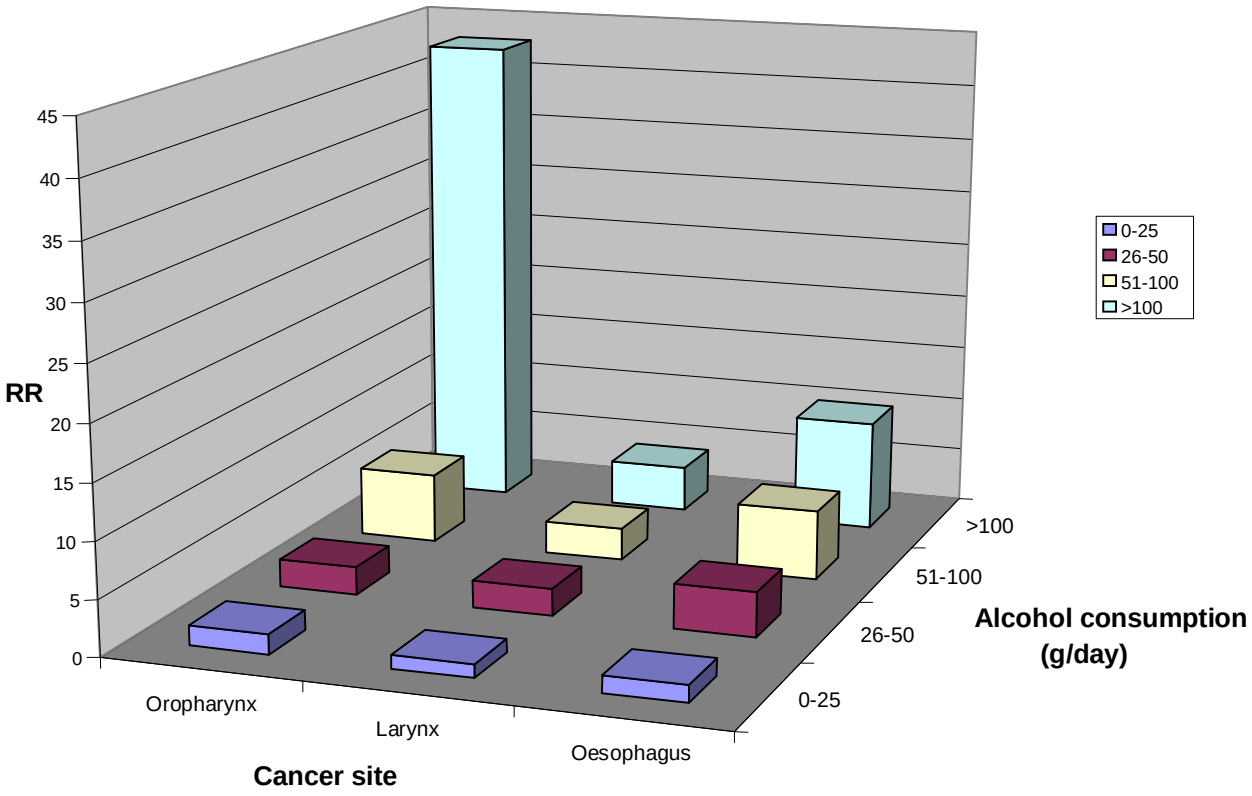


Figure 1. Effect of alcohol consumption on oropharyngeal, laryngeal and oesophageal cancer risks expressed as relative risks (RR). Data were collected and modified from the reviewed publications.

1.2 Synergistic effect of alcohol and tobacco on upper digestive tract cancers

Search terms:
Alcohol drinking AND * smoking
AND
mouth neoplasms (aetiology, epidemiology)
OR
pharyngeal neoplasms (aetiology, epidemiology)
OR
laryngeal neoplasms (aetiology, epidemiology)
OR
oesophageal neoplasms (aetiology, epidemiology)
OR
stomach neoplasms (aetiology, epidemiology) [239]

To cover all meta-analyses and the most recent studies, a search was also performed in PubMed (restricted to the years 2001-2005) with the following terms:
- Alcohol drinking AND smoking AND cancer AND meta-analysis [11]
- Alcohol drinking AND smoking AND cancer AND combined [55].

As a result, 305 publications were reviewed by abstract and were included in the review if the following criteria were met: meta-analysis, cohort or case-control study published as an original article, findings expressed as ORs or RRs and the combined effect of alcohol drinking and smoking specifically reported.

Altogether 85 publications met the inclusion criteria. Of the included studies, 78 were case-control studies, four cohort studies and three meta-analyses. These meta-analyses from Bagnardi, Zeka and Altieri cover the published literature for different databases (Medline, Current contents, etc) to the year 2000 (Bagnardi et al., 2001a), 2001 (Zeka et al., 2003) and 2005 (Altieri et al., 2005), respectively. More detailed descriptions of these publications are given in the preceding section.
The IARC has published an up-to-date monograph of the IARC working group meeting on June 2002 concerning tobacco smoking (IARC, 2004). An extensive summary is included in the IARC monograph covering all relevant publications (to 2001) concerning the combined effects of alcohol drinking and tobacco smoking on cancers of the upper aerodigestive tract (IARC, 2004).

To avoid an excessive number of references, only the three meta-analyses, IARC monograph and publications from the year 2002 were included in this review. Thus, three meta-analyses, two cohort studies and 14 case-control studies were reviewed. Both cohort studies are Japanese, one reporting the risk effect of smoking and alcohol drinking on oesophageal cancer (Sakata et al., 2005) and the other on stomach cancer (Kikuchi et al., 2002).

Alcohol and tobacco are the best-recognized and unquestioned risk factors in developed countries for oral, pharyngeal, laryngeal and oesophageal cancers. Alcohol and tobacco enhance each other's effects on the risk for the above-mentioned cancers. It has been estimated that due to this synergism over 75 % of cancers of the upper digestive tract are attributable to alcohol and tobacco, and therefore, avoiding tobacco and alcohol could prevent the majority of cases (Bagnardi et al., 2001a). Despite smoking and drinking being known risk factors,

separating their independent effects remains difficult since heavy drinkers tend to be heavy smokers, and vice versa. Furthermore, studies often include too few cases who neither smoke nor drink (Altieri et al., 2002). Nevertheless, according to most of the epidemiological studies, the joint effect of alcohol consumption and tobacco smoking on cancer risks of the oral cavity, pharynx and oesophagus follow the multiplicative model, i.e. the combined effect of both of these agents is greater than simply adding the effects together.

1.2.1 Mouth and pharynx

The RR for cancers of the oral cavity and pharynx, including the synergistic effect of alcohol and smoking for the highest exposure levels of both risk factors, ranges in meta-analyses from 21.2 (Zeka et al., 2003) to 51 (Castellsague et al., 2004). The latest IARC review reports the respective RRs to range from 5 to 77.3. The huge variation in risk is due to the different consumption levels reported in the studies. The highest level of tobacco use reported to range from >10 g (of tobacco)/week to >40 cigarettes/day (a cigarette weighs approximately 1 g with the tobacco content varying between 65 % and 100 %, depending on the type of cigarette) and alcohol consumption from >20 g/day to 85 drinks/week (one drink is about 10 g of ethanol). There are also discrepancies between some studies, e.g. the RR for oral cancer in non-drinking heavy smokers may be 1 or less which is the opposite reported in the majority of the studies considering cancer risks at the same anatomical site (IARC, 2004). The independent effect (RR) of smoking on non-drinking subjects for oral cavity or pharyngeal cancer ranges from 1.85 to 13.6 (Castellsague et al., 2004; Huang et al., 2003). All studies confirm the synergistic effect of alcohol use and smoking on oral and pharyngeal cancer risk (Bagnardi et al., 2001a; Castellsague et al., 2004; Huang et al., 2003; IARC, 2004; Lissowska et al., 2003; Zeka et al., 2003).

1.2.2 Larynx

Most of the case-control studies on laryngeal cancer provide strong evidence for the synergistic effect of alcohol consumption and smoking (IARC, 2004). However, the meta-analysis of Bagnardi et al., suggests that the combined and independent effects of alcohol and tobacco smoking on laryngeal cancer are somewhat inconsistent compared with oropharyngeal and oesophageal cancers (Bagnardi et al., 2001b). This is most probably due to the glottic and subglottic regions of the larynx anatomically being exposed to inhaled agents. Thus, smoking has a more pronounced effect on the cancer risk in some areas. The independent effect on the OR for cancer at the highest level of smoking alone ranged in the reviewed publications from 7.7 to 18.9 (Talamini et al., 2002; Zeka et al., 2003). When alcohol consumption (highest level) was combined with smoking, the RR increased multiplicatively. A case-control study of women (68 cases/340 controls) reports a very high OR for laryngeal cancer risk. Subjects who smoked >15 cigarettes/day and consumed > 3 alcoholic drinks/day had an OR of 317 (Gallus et al., 2003). The highest RR for combined action of alcohol and tobacco is reported in Guenel's study (411 cases/4135 controls). The consumption of >160 g/day of alcohol

combined with the highest smoking category (>30 g/day) yields an RR for glottic and supraglottic cancers of 289 and 1094, respectively (IARC, 2004). These very high RRs might be due to too few cases and/or controls in these studies. However, these results are consistent with the multiplicative risk model of the combined effect of alcohol and tobacco smoking on laryngeal cancer. In Altieri's meta-analysis including 20 studies, the risk estimate for laryngeal cancer for the highest level of consumption for both factors as compared with the lowest one were from 8.0 to over 100, and a multiplicative risk model was indicated (Altieri et al., 2005). Also the meta-analysis of Zeka et al., suggests a multiplicative nature – at least more than additive – of the joint exposure to alcohol and tobacco (Zeka et al., 2003). Case-control studies from India and France conclude that the joint effects of smoking and drinking are greater than additive (Menvielle et al., 2004; Znaor et al., 2003). In the review of IARC, only one study from the year 1992 (Zheng et al., 1992) reported evidence inconsistent with the synergistic effect of alcohol and smoking.

1.2.3 Oesophagus

Both meta-analyses (Bagnardi et al., 2001b; Zeka et al., 2003), and three of the four case-control studies (513 cases/818 controls); (Lee et al., 2005b), (805 cases/3461 controls); (Garavello et al., 2005), (1248cases/1248 controls); (Ke et al., 2003) and the cohort study (42 578 subjects); (Sakata et al., 2005) concluded that a synergistic interaction exists between alcohol consumption and smoking with regard to oesophageal cancer risk. One case-control study from Taiwan (309 subjects) found no association between alcohol consumption and oesophageal cancer risk (Wu et al., 2003). This conclusion was, however, based on subjects being defined as alcoholics with alcohol consumption as low as one dose per week, which is far from abuse. The seven case-control studies and one cohort study included in the IARC review support the joint effect of smoking and alcohol use and conclude that the risk seems to be greater than additive (IARC, 2004). In addition, the first analysis of the EPIC study with 412 000 cases of oesophageal cancer confirms that there is a multiplicative effect on cancer risk with concomitant exposure to heavy drinking and smoking (Boeing and EPIC Working Group on Dietary Patterns., 2002) (not included in this review because of unfinished analysis). Based on the reviewed studies, the highest combined risks for the highest levels of alcohol use and tobacco smoking range from 4.3 (Ke et al., 2003) to 149 (IARC, 2004).

The IARC monograph also reports findings from nine case-control studies and one cohort study on cancers of the "mixed upper aerodigestive tract", including subjects with squamous cell carcinomas on a non-specific site in the head and neck regions. These studies provide strong evidence for the synergistic action of smoking and alcohol consumption on cancer risk (IARC, 2004). The results regarding the synergistic and multiplicative effect of smoking and alcohol on mixed upper aerodigestive tract cancers are summarized in the Figure 2, which includes six case-control studies reporting at least three smoking categories and three alcohol consumption categories in non-Oriental populations (Andre et al., 1995; Baron et al., 1993; Franceschi et al., 1990;

Kabat et al., 1994; Maier et al., 1992; Mashberg et al., 1993).

1.2.4 Stomach

No consistent data are available about the independent effect of alcohol consumption on gastric cancer. Hence, one cannot conclude anything about the synergism of tobacco and alcohol on stomach cancer risk. Furthermore, none of the reviewed publications report a possible synergistic or antagonistic effect.

Nevertheless, we can conclude that there are numerous cohort and case-control studies proving the causal role of tobacco smoking in the development of stomach cancer (not systematically reviewed). The RR for stomach cancer for the highest level of tobacco use (~20 cigarettes/day) varied according to 29 cohort and 45 case-control studies from 0.7 to 12 (IARC, 2004).

In conclusion, there is strong, grade A level of evidence indicating a significant synergistic effect of alcohol and smoking on all upper digestive tract cancers except stomach cancer. Furthermore, data clearly support the multiplicative effect of alcohol and tobacco on cancer risk. Depending on the anatomical site, the multiple-risk effect varies, being in any case marked.

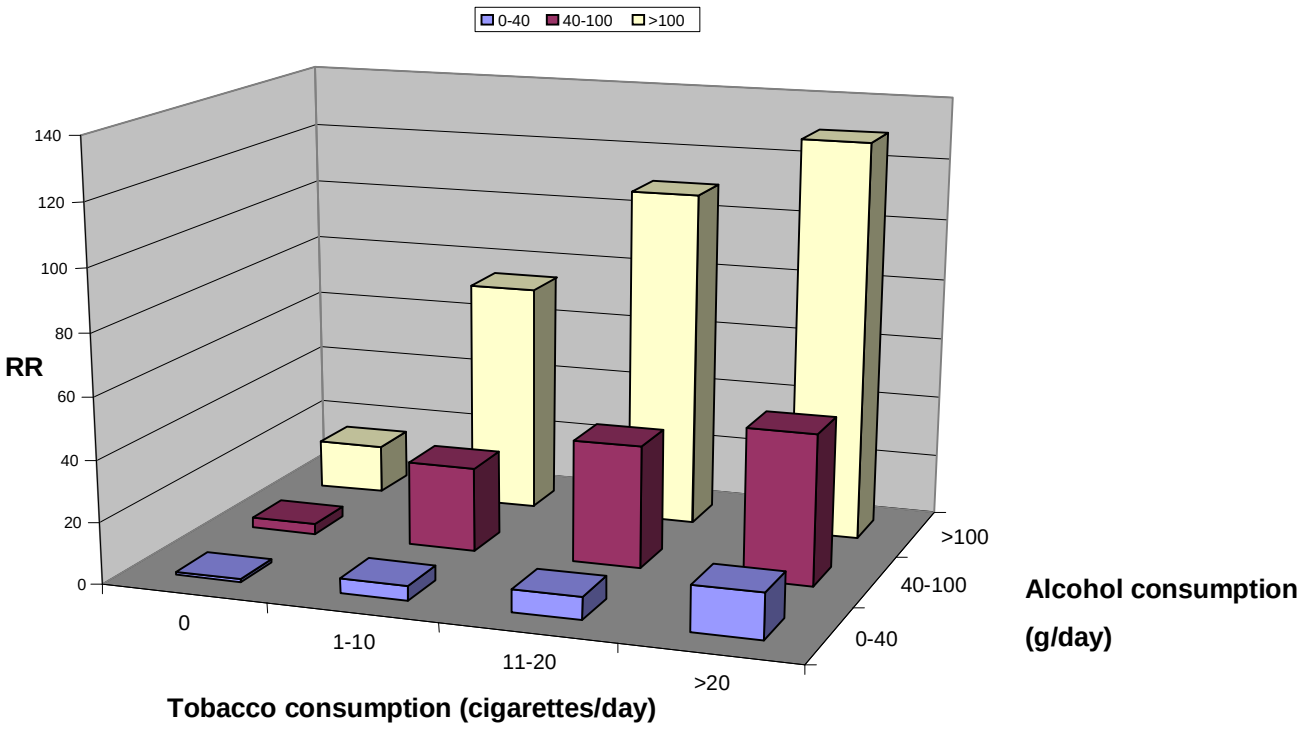


Figure 2. Multiplicative risk effect of smoking and alcohol consumption (expressed as relative risk, RR) on mixed upper aerodigestive tract cancers.

1.3 Effect of gene polymorphism on alcohol-related upper digestive tract cancers

There is strong recent epidemiological evidence suggesting that the risk of alcohol-related upper aerodigestive tract cancers is markedly influenced by either a genetically reduced ability to eliminate the first metabolite of alcohol oxidation acetaldehyde or an enhanced ability to produce it. Therefore, two systematic literature searches were carried out on this topic.

1.3.1 ALDH-polymorphism

A search from MEDLINE [*Alcohol drinking OR alcoholism AND aldehyde dehydrogenase AND neoplasm (aetiology/epidemiology)] resulted in 33 studies of which 13 case-control studies, (over 3245 subjects) examined upper digestive tract cancers. In addition, two studies and one meta-analysis (7 studies with 905 cases); (Lewis and Smith, 2005) were manually located. All but one (Katoh et al., 1999) of the studies confirmed increased upper digestive tract cancer risk associated with alcohol consumption and the low-activity form of ALDH2. In Yokoyama's study, the RRs in ALDH2-deficient subjects, after adjustment for confounders, for oropharyngolaryngeal, oesophageal, stomach, colon and lung cancers were 11.1, 12.5, 3.5, 3.4 and 8.2, respectively, compared with individuals with normal enzyme activity (Figure 3) (Yokoyama et al., 1998).

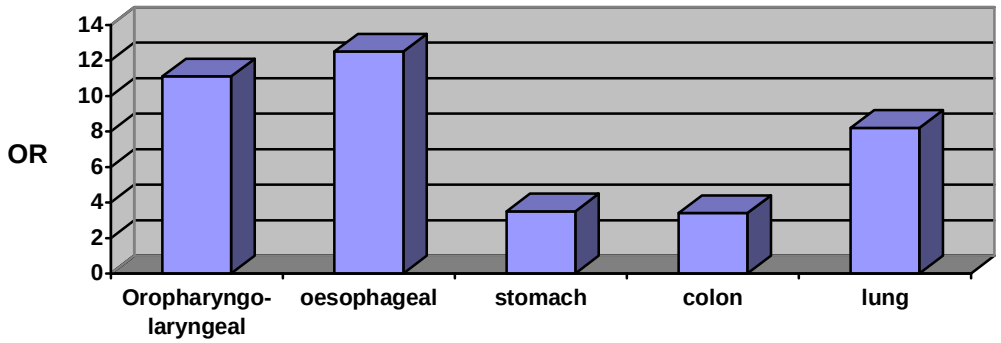


Figure 3. Increased risks for oropharyngolaryngeal, oesophageal, stomach, colon and lung cancers (expressed as odds ratios, OR) in ALDH2-deficient subjects when compared with individuals with normal enzyme activity (Modified from *Yokoyama A et al., Carcinogenesis 1998;19:1383-1387*).

Among Japanese male alcoholics, a strong association have been noted between inactive heterozygous ALDH2 and cancer of the oral cavity/oropharynx (OR, 20.8) and hypopharynx/epilarynx (OR, 28.9) (Nomura et al., 2000; Yokoyama et al., 1998; Yokoyama et al., 2001). In the presence of the ALDH2*2 allele, the risk of field cancerization is also markedly increased (Morita et al., 1994; Schwartz et al., 1994; Yokoyama et al., 1996b; Yokoyama et al., 2002). This multiplicity of upper aerodigestive tract cancers suggests a common aetiology, thus supporting the role of acetaldehyde in the carcinogenesis.

This conclusion is also supported by a recent meta-analysis of alcohol, ALDH2 and oesophageal cancer, including seven studies carried out in Japan, Taiwan and Thailand. A significantly increased oesophageal cancer risk (OR, 3.19) was found in ALDH2*1/*2 heterozygotes, which provides evidence for the carcinogenic action of acetaldehyde (Lewis and Smith, 2005). Already one of the very first studies in which the association of increased cancer risk related to acetaldehyde was discovered showed increased oesophageal cancer risk in alcoholics with the ALDH2*2 allele (Yokoyama et al., 1996a).

1.3.2. ADH polymorphism

An ALDH2 deficiency is extremely rare in the Caucasian population. However, a polymorphism in the ADH enzyme may have an increasing impact on the capacity to produce acetaldehyde.
A MEDLINE search [*alcohol drinking OR alcoholism AND alcohol dehydrogenase AND neoplasms (aetiology, epidemiology, genetics)] yielded 29 studies, 12 of which were included here as they covered upper digestive tract cancer risk and the ADH1C allele (former ADH3). In a recent pooled analysis including 1325 cases, the ADH1C*1 allele was concluded not to confer an increased risk for head and neck cancers (Brennan et al., 2004). This analysis missed, however, the two most recent studies (Homann et al., 2005; Visapää et al., 2004) in which a positive association was found between the ADH1C*1 allele and the risk of oesophageal, head and neck cancers. The highest ORs for upper digestive tract cancers were reported in a study from Puerto Rico: 40.1 and 7.0 for homozygotic and heterozygotic alcoholics, respectively for the ADH1C*1 allele (Harty et al., 1997). A positive association was also found in a French study (Coutelle et al., 1997). However, six other studies reported conflicting results (Bouchardy et al., 2000; Olshan et al., 2001; Risch et al., 2003; Schwartz et al., 2001; Sturgis et al., 2001; Zavras et al., 2002). In conclusion, the association of ADH1C with alcohol-related upper digestive tract cancer risk is under debate, and further studies on this topic are needed.

The IARC monograph on acetaldehyde also pays attention to some (published before 1999) epidemiological studies reporting increased cancer risk with ALDH2 deficiency or the ADH1C*1 allele. Acetaldehyde was concluded to possibly be carcinogenic to humans (IARC, 1999). Moreover, acetaldehyde has been classified as “reasonably anticipated to be a human carcinogen” by the US government’s 9th report on carcinogens (U.S. Department of Health and Human Services, P.H.S., National Toxicology program, 2001

2 ETHANOL METABOLISM IN THE GASTROINTESTINAL TRACT

A brief review of distribution and metabolism of ethanol is given in this section.

2.1 Distribution of ethanol

Ethanol is absorbed without transport mechanisms, by simple diffusion from the digestive tract because of its small molecular size and good water solubility (Crabb et al., 1987; Wallgren and Barry III, 1970). Of the ingested ethanol, 70-80 % is absorbed from the proximal small intestine – the duodenum and the upper jejunum. A smaller proportion (~25 %) is absorbed from the stomach. After absorption, ethanol is distributed via blood circulation and diffusion throughout the body fluids. Distribution of alcohol is mainly related to the water content of various organs and tissues. Consequently, ethanol concentrations after the distribution phase in the digestive tract, e.g. in the ileum, (Halsted et al., 1973) colon (Levitt et al., 1982) and oral cavity (Jones, 1979) are equal to those in the blood. The high water content of saliva and urine might even result in a slightly higher ethanol concentration in saliva than in blood (Bendtsen et al., 1999; Jones, 1979).

Most of the ethanol (90-95%) is completely metabolized in the body by oxidation and excreted as carbon dioxide (CO_2) and water. A minor portion of ingested ethanol is excreted unaltered via urine, sweat and breath (Holford, 1987).

2.2 Ethanol and acetaldehyde metabolism

The liver is the main site for ethanol metabolism. Under normal conditions, the liver eliminates 75-90% of ethanol (Agarwal and Goedde, 1990). However, in severe hepatic cirrhosis, extrahepatic elimination of ethanol can account for up to 40% (Utne and Winkler, 1980). Three metabolic pathways are available for ethanol oxidation in the liver: cytosolic alcohol dehydrogenase (ADH), microsomal ethanol oxidizing system (MEOS) and catalase; the ADH pathway is the most important and effective (Figure 4). All of these pathways yield acetaldehyde as an end-product. Acetaldehyde is further converted to acetate, mainly by mitochondrial aldehyde dehydrogenase.

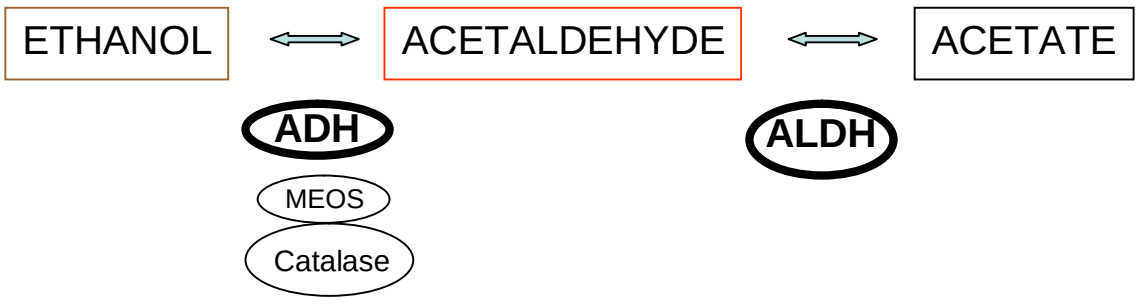


Figure 4. Metabolic pathways for ethanol and acetaldehyde oxidation.

2.2.1 Alcohol dehydrogenase

ADH catalyses the reversible, NAD-dependent oxidation of many alcohols to the corresponding aldehydes. In the case of ethanol, the reaction is as follows:

$CH_3CH_2OH + NAD \leftrightarrow CH_3CHO + NADH + H$

ADH is abundant in the liver and its physiological role has been postulated to be the degradation of low levels of endogenous ethanol produced by microbial fermentation in the gut. Another possible role is the degradation of endogenous steroids (Krebs and Perkins, 1970). Human ADHs can be grouped into five classes, I-V, based on the characteristics of their primary structure (Jörnvall and Höög, 1995). However, the most important enzymes in hepatic ethanol elimination are class I ADHs. These enzymes, with both a low Km (~1 mM) and a high Vmax for ethanol, are responsible for the bulk of ethanol oxidation from the blood (Blair and Vallee, 1966).Class I ADHs have three isoenzymes: ADH1A, ADH1B and ADH1C, which are expressed by three genes. ADH1B and ADH1C are polymorphic; for ADH1B, three different (ADH1B*1,-*2,-*3) and for ADH1C (ADH1C*1,-*2) two different allelic forms have been found (Bosron and Li, 1986; Duester et al., 1999). The clinical significance of and interest in human ADHs is based on differences in the ethnic and racial distribution of these allelic forms. For example, the frequency of the ADH1C*1 allele is approximately 50-60% in Caucasians and more than 90% in Asians (Bosron and Li, 1986). Furthermore, the enzyme activities encoded by these different allelic forms differ from each other; individuals with the ADH1C*1/*1 genotype are, for instance, able to metabolize ethanol to acetaldehyde, at least *in vitro* 2.5 times faster than individuals with other ADH1C genotypes (Bosron and Li, 1986). This genotype is more prone to digestive tract cancers, as discussed earlier, probably due to the above-mentioned genetic characteristics, which are discussed further in sections 3.3 and 4.2.

The other ADH classes (II-V) in humans are expressed in the mucosa covering the human digestive tract. The mucosa of the gingiva and tongue expresses class III and class IV ADH isoenzymes (Dong et al., 1996). The main ADH isoenzyme in the oesophagus is class IV (Yin et al., 1993). The stomach expresses many ADH isoenzymes, of which classes I and IV are postulated to be the most important (Pares et al., 1992; Seitz and Oneta, 1998; Yin et al., 1993). In conclusion, it can be generalized that class IV ADH is characteristic of the upper digestive tract and class I of the rest of the intestinal tract. The Km values for the above-mentioned ADHs range from 1 to 40 mM, the highest values being in the upper digestive tract and the lowest in the large intestine (Seitz and Oneta, 1998). This is logical as these ethanol concentrations correspond to the *in vivo* amounts to which the mucosa is exposed. While the exact *in vivo* role of mucosal ADH remains obscure, it is presumably involved in the regulation of local acetaldehyde concentration in the digestive tract (Visapää et al., 2002).

2.2.2 MEOS

In humans, estimations for the contribution of the microsomal ethanol oxidizing system (MEOS), the CYP2E1-cytochrome fraction,

to the total ethanol metabolism vary widely, from 3% to over 30% (Alderman et al., 1987; Handler et al., 1988). It is suggested that the MEOS contributes to ethanol elimination only at higher blood ethanol levels since its Km for ethanol is 7-10 mM. CYP2E1 may, however, adapt to the constantly high blood ethanol concentration in chronic alcohol consumers (Lieber, 1988). The MEOS oxidizes ethanol to acetaldehyde as follows:

$CH_3CH_2OH + NADPH + H + O_2 \rightarrow CH_3CHO + NADP+ 2H_2O$

2.2.3 Catalase

Catalase, located in peroxisomes, can oxidize ethanol to acetaldehyde when hydrogen peroxide is available as follows:

$CH_3CH_2OH + H_2O_2 \rightarrow CH_3CHO + 2H_2O$

Since the reaction is limited by the bioavailability of hydrogen peroxide and its production in the liver is rather low, catalase plays only a minor role < 2% in hepatic ethanol metabolism (Boveris et al., 1972).

2.2.4 Aldehyde dehydrogenase

Regardless of the pathway by which ethanol is oxidized, acetaldehyde is the first metabolic product. The second reaction in ethanol metabolism is the oxidation of acetaldehyde to acetate by the aldehyde dehydrogenase (ALDH) enzyme. In humans, many ALDH isoenzyme classes have been isolated. The isoenzyme mainly responsible for acetaldehyde oxidation is the mitochondrial class II ALDH (ALDH2), which has a micromolar Km value and a high affinity for acetaldehyde (Lands, 1998). Human ALDH2 enzyme is polymorphic, with two allelic forms, ALDH2*1 and ALDH2*2. The ALDH2*2 allele is the result of a point mutation in the normal allele. Individuals homozygous (ALDH2*2/*2) for this mutated allele lack ALDH activity, whereas heterozygous individuals (ALDH2*1/*2) have 30-50% of the activity of ALDH2*1 homozygotes (Crabb et al., 1989). This mutation is extremely rare in Caucasians but frequent in Asians; for example 50% of the Japanese population is ALDH2-deficient (Agarwal and Goedde, 1992).

The homozygous form of inactive ALDH2 offers protection against alcoholism. This is most probably due to the accumulation of acetaldehyde during alcohol metabolism, which causes aversive symptoms (Peng et al., 1999). Heterozygote subjects can, however, tolerate the unpleasant symptoms caused by increased acetaldehyde levels, and thus can drink alcohol or even develop alcohol dependency (Wall et al., 1992).

Blood acetaldehyde levels in these heterozygotes have been shown to be between 8 and 24 µM, even after a very low dose (0.1 g/kg of body weight) of ethanol (Enomoto et al., 1991). Most importantly, acetaldehyde concentration in the saliva of these subjects is also elevated after ethanol challenge due to diminished elimination of acetaldehyde in the parotid glands (Väkeväinen et al., 2000). By comparison, normal healthy subjects have very low levels (<0.5 µM) of acetaldehyde in their blood during ethanol oxidation (Eriksson and Fukunaga, 1993), and their salivary acetaldehyde is only of microbial origin

(Väkeväinen et al., 2001). As the mutation in the ALDH2 gene results in impaired local acetaldehyde metabolism, and consequently, in increased acetaldehyde exposure after alcohol consumption ALDH2*2 heterozygotic subjects can be considered partial knock-out models for the effects of high levels of acetaldehyde. This heterozygocity together with heavy drinking induces a markedly increased risk for gastrointestinal tract cancers compared with individuals possessing the normal ALDH2 genotype (Yokoyama and Omori, 2003; Yokoyama et al., 1998), as discussed earlier and further described in sections 3.3 and 4.2.

2.3 Microbial ethanol metabolism

In addition to the liver, microbes in the digestive tract are able to engage in ethanol oxidation. To produce energy under anaerobic conditions, microbes may degrade sugars to ethanol. The last step in this alcoholic fermentation is the reduction of acetaldehyde to ethanol, catalysed by bacterial ADH (Reid and Fewson, 1994). This has been described in detail for *Escherichia coli* (Clark, 1989), group N *Streptococci* (Lees, 1976) and *Enterobacteriaceae* in general (Salveson and Bergan, 1981). Due to ongoing microbial reactions, especially in the large intestine, measurable amounts of ethanol are formed in the gastrointestinal tract. This endogenous ethanol is partly dissolved in the portal blood circulation and thereafter efficiently metabolized in the liver, never reaching the systemic blood circulation (Krebs and Perkins, 1970). However, under some conditions associated with microbial overgrowth, elevated endogenous ethanol levels are detected even in blood (Kaji et al., 1984).

An ADH-mediated reaction is reversible also in microbes (Maconi et al., 1988). This means that if oxygen and excessive ethanol are present, the reaction can proceed in the opposite direction, with ethanol being oxidized to acetaldehyde. There are numerous *in vitro* and *in vivo* studies providing evidence for microbially mediated acetaldehyde production in humans. Nosova et al., for example characterized the ADH activity and acetaldehyde production *in vitro* of several bacteria representing human microbiota (Nosova et al., 1997; Nosova et al., 2000). Many different strains of faecal *E. coli* and other Gram-negative rods, mainly belonging to the *enterobacteriaceae* family, produce significant amounts of acetaldehyde when incubated aerobically *in vitro* in the presence of ethanol (Jokelainen et al., 1996). Human colonic contents (mixture of colonic bacteria) produce acetaldehyde from physiological levels of ethanol in a dose-dependent manner (Jokelainen et al., 1994). *In vivo*, ethanol administration to pigs leads to a marked increase in intracolonic acetaldehyde concentration (Jokelainen et al., 1996b). Similarly, high acetaldehyde levels are detected in the caecal samples of rats after an acute dose of ethanol (Visapää et al., 1998). The microbially mediated pathway for ethanol metabolism can be modulated by treatments with antibiotics or prebiotics. Ciprofloxacin decreases the number of aerobic bacteria in the large intestine and this is associated with a significant decrease in ethanol elimination rate and intracolonic acetaldehyde production both in experimental animals and in humans (Nosova et al., 1999; Tillonen et al., 1999b).

Similarly, lactulose decreases intracolonic pH dependent ADH-mediated acetaldehyde production in rats (Zidi et al., 2003). Ethanol-derived acetaldehyde production occurs also in the achlorhydric stomach caused by atrophic gastritis or protein pump inhibitors, which leads to bacterial overgrowth in the neutral stomach (Väkeväinen et al., 2000; Väkeväinen et al., 2002).

In the upper digestive tract, significant microbially mediated acetaldehyde production is detected when human mouth and bronchopulmonary washings are incubated with ethanol *in vitro* (Jauhonen et al., 1982; Miyakawa et al., 1986; Pikkarainen et al., 1981). Furthermore, the mouth washings of patients with oropharyngeal cancer produce higher amounts of acetaldehyde *in vitro* than those of healthy controls (Jokelainen et al., 1996a). Increased *in vitro* acetaldehyde production from ethanol is also observed in heavy drinkers and smokers (Homann et al., 2000). Smoking, alcohol consumption, poor oral hygiene and differences in oral microbial flora may thus influence local levels of acetaldehyde in saliva and consequently the whole upper digestive tract. The role of these key factors in the regulation of local acetaldehyde production in the upper digestive tract will be discussed in the next section.

3 ETHANOL AND ACETALDEHYDE AS CARCINOGENIC SUBSTANCES

3.1 Ethanol

Although the consumption of alcoholic beverages is an established risk factor for digestive tract cancers, there is no evidence that ethanol itself is a carcinogen. Systematic reviews have generally stated that pure ethanol is not carcinogenic in laboratory experiments (Doll et al., 1999; IARC, 1988). However, many of the studies described in the literature cannot be used for the evaluation of carcinogenicity of alcohol due to limitations in experimental design (IARC, 1988). Based on limited data available there is no experimental evidence that alcohol itself is a carcinogen (Doll et al., 1999).

On the other hand, evidence has emerged that ethanol may act as a co-carcinogen in the production of cancers. In the IARC monograph, a large number of animal studies indicate that ethanol modifies or enhances the carcinogenic potential of known carcinogens (IARC, 1988). Many more or less indirect mechanisms by which chronic alcohol consumption may stimulate carcinogenesis also exist. However, this topic is not systematically reviewed or discussed in detail in this thesis. In brief, these potential mechanisms include (i) local direct mucosal damage and actions of alcohol as a solvent for carcinogenic compounds (Albanes and Winick, 1988; Blot, 1992; Cohen and Ellwein, 1990; Maier et al., 1986; Salo, 1983), (ii) induction of cytochrome P-4502E1, which produces free radicals and activates precarcinogens (Albano and Clot, 1996; Eskelson et al., 1993; Seitz et al., 1998), (iii) nutritional deficiencies in heavy drinkers (Seitz and Suter, 2002; Seitz et al., 1998), (iv)

alterations in the metabolism of retinal and retinoic acid (Pöschl and Seitz, 2004), (v) congeners in alcoholic beverages possible acting as mutagens (Lieber et al., 1986), (vi) immunosuppression induced by chronic alcoholism (Palmer, 1978; Pöschl and Seitz, 2004), (vii) alterations in hormone status, e.g. increased oestradiol levels in women (Singletary and Gapstur, 2001) and (viii) interaction of alcohol and/or acetaldehyde with folate metabolism. The antagonist effect of alcohol on folate status has been known for two decades (Halsted et al., 2002; Hillman and Steinberg, 1982). The risk of colon cancer has also been shown to be higher in persons with low folate status and high alcohol consumption than in alcohol-consuming persons with high folate status (Giovannucci et al., 1995; La Vecchia et al., 2002). There are several mechanisms that might contribute to the folate deficiency associated with alcohol consumption and cancer. One of these is the ability of acetaldehyde to cleave folate (Shaw et al., 1989). Accordingly, local acetaldehyde production by microbes in the large intestine might contribute to the risk of mucosal neoplasia (Giovannucci, 2004; Homann et al., 2000).

In conclusion, the lack of carcinogenicity of alcohol itself in animal studies and the failure of studies to fully elucidate the mechanisms by which ethanol acts as a co-carcinogen provide evidence for the important role of acetaldehyde in inducing carcinogenicity of alcohol consumption.

3.2. Acetaldehyde

In most of the *in vitro* studies with human and other mammalian cells, ethanol alone has been shown not to induce DNA damage, sister chromatid exchanges or chromosomal aberrations (IARC, 1988). On the other hand, sister-chromatid exchanges are seen *in vitro* when the alcohol dehydrogenase enzyme is added together with ethanol to the experimental system, resulting in the production of acetaldehyde (Obe et al., 1986).

Acetaldehyde is a highly toxic and reactive compound, which has been linked to several toxic effects associated with heavy drinking. In addition to a large number of *in vitro* and *in vivo* experimental studies, there is increasing epidemiological and biochemical evidence regarding the carcinogenicity of acetaldehyde in humans due to impaired or enhanced acetaldehyde metabolism. To elucidate the role of ethanol-derived acetaldehyde in the carcinogenicity of alcohol drinking, a systematic review on the mutagenic and toxic effects of acetaldehyde was executed.

A MEDLINE search with the terms: *acetaldehyde (toxicity) OR *acetaldehyde AND mutagenesis OR metaplasia resulted in 36 publications. To also cover the very latest studies, a search was performed in PubMed (limited to publications from 1999 onwards) with the terms acetaldehyde AND mutagenesis OR carcinogenesis [78].
The IARC has also reviewed the carcinogenicity data of acetaldehyde in 1985, 1987 and 1999. Thus, to avoid an enormous number of references, this review was limited to the IARC monograph and to the studies published after that (i.e. from 1998 onwards). All studies published as an original article were included. Studies reporting neurotoxicity, teratogenesis of acetaldehyde or tobacco smoke-derived

acetaldehyde were excluded, as they are discussed in the section 3.3. The search was complemented with manual searches of references in other published articles when necessary.

3.2.1 Acetaldehyde-related experimental toxicity

The IARC monograph contains 56 *in vitro* studies concerning the harmful genetic effects of acetaldehyde. These include test systems in which acetaldehyde was tested with bacteria, or with human or animal cells. Thirty-seven of these studies concluded that acetaldehyde had harmful effects. The majority of the reverse mutation tests in which *Salmonella typhimurium* or *Escherichia coli* were incubated with acetaldehyde (concentrations 55 – 200 µM) failed, i.e. a reverse mutation was not induced. This test is, in general, used to detect possible point mutations induced by chemicals.
However, a weak, positive, forward mutation (a mutation that converts a wild-type allele to a mutant allele) was detected in *Saccharomyces cerevisiae* with 0.5 mM acetaldehyde. It should be noted that the *in vitro* bacterial mutation test cannot provide direct information about the mutagenic potency of a substance in mammals.

Acetaldehyde as a toxic compound has been suggested to induce the expression of acetaldehyde-detoxifying genes in microbes, leading to acetaldehyde trapping with e.g. sulphur-containing amino acids. Thus, microbes in the ethanol/acetaldehyde-containing environment can tolerate acetaldehyde (Aranda and del Olmo, 2004). On the other hand, acetaldehyde (4.4 and 4.5 mM concentrations, respectively) induced chromosome malsegregation in *Aspergillus nidulans* (Crebelli et al., 1989) and was mutagenic in *Drosophilia melanogaster* (Woodruff et al., 1985).

In studies with mammalian cells, acetaldehyde induced DNA-protein cross links to rat nasal mucosal cells, sister chromatid exchanges in hamster ovary cells and mouse bone-marrow cells both *in vitro* and *in vivo*. Also aneuploidy and chromosomal aberrations were detected in mammalian fibroblasts *in vitro*. These mutagenic effects occurred at acetaldehyde concentrations from 30 µM to 1000 µM. There are numerous studies in which sister chromatid exchanges (i.e. reciprocal exchanges of DNA between two sister chromatids of a duplicating chromosome) have been detected in human lymphocytes *in vitro* at very low acetaldehyde concentrations (~88 µM) (IARC, 1999). Furthermore, gene mutations and chromosomal aberrations are induced with 0.2-0.3 mM acetaldehyde. Acetaldehyde caused DNA strand breaks and cross links in human lymphocytes *in vitro*, which were not, however, detected in human bronchial epithelial cells (IARC, 1999). When human lymphocytes and digestive tract mucosal cells are exposed to ethanol and acetaldehyde *in vitro*, cell viability decreases and DNA strand breaks are observed; however, the specific effects of ethanol or acetaldehyde cannot be clearly determined (Blasiak et al., 2000).

Evidence from alcoholics and experimental animal models indicates that acetaldehyde and aldehydic products of lipid peroxidation can bind to proteins, forming many types of protein adducts, including those with

acetaldehyde, malondialdehyde, malondialdehyde-acetaldehyde hybrids, 4-hydroxynonenol and hydroxyethyl radicals (Freeman et al., 2005). Adduct formation may lead to several adverse consequences, such as interference with protein function, stimulation of fibrogenesis and induction of immune responses (Niemelä, 1999). As stated earlier, ethanol is oxidized to acetaldehyde also by the microsomal ethanol oxidizing system (MEOS). This results in the production of reactive oxygen metabolites and in oxidative stress. Acetaldehyde can also induce lipid peroxidation. The mechanism underlying this has been hypothesized to be acetaldehyde's capacity to reduce hepatic glutathione levels (Shaw et al., 1981; Situnayake et al., 1990). Lipid peroxidation can generate aldehydes, 4-hydroxynonenal and malondialdehyde. These reactive products in addition to acetaldehyde can then interact with amino acids, attacking the nucleophilic groups of proteins to form stable or unstable adducts (Niemelä et al., 2002; Niemelä, 2001; Tuma, 2002). The formation of acetaldehyde-protein adducts is best described in the liver, alcoholic cardiomyopathy and alcoholic skeletal muscle myopathy (Niemelä, 1999; Patel et al., 2005; Thiele et al., 2004). Their formation *in vivo* and the relative importance in carcinogenesis remain largely unclear (Freeman et al., 2005).

Acetaldehyde also interacts with DNA, producing DNA adducts. The covalent binding of acetaldehyde to DNA and the formation of stable adducts is one mechanism by which it could induce replication errors or mutations in oncogenes or tumour-suppressor genes. This is considered to be a critical event in chemical carcinogenesis related to alcohol consumption (Vaca et al., 1995). The best-studied acetaldehyde-DNA adduct is N-ethyl-2-deoxyguanosine. This adduct is detected *in vitro* when calf thymus or pure DNA is incubated with millimolar concentrations of acetaldehyde (Fang and Vaca, 1995). This adduct is also found in the DNA of human buccal cells following exposure to acetaldehyde as well as in human urine and white cells of alcohol abusers *in vivo* (Brooks and Theruvathu, 2005; Fang and Vaca, 1997; Matsuda et al., 1999; Vaca et al., 1998). Furthermore, three stable DNA adducts are formed when calf thymus is incubated with rather high, non-physiological acetaldehyde concentrations (up to 40 mM) (Wang et al., 2000). The contribution of these DNA adducts to cytogenetic abnormalities is unclear since data with regard to adduct-induced errors in DNA replication and synthesis are controversial (Matsuda et al., 1999; Perrino et al., 2003; Terashima et al., 2001).

Acetaldehyde may also inhibit the function of the human DNA repair enzyme, 0^6 methyl-guanine transferase, *in vitro* and *in vivo* already at a 0.01 mM concentration (Espina et al., 1988). Concentrations of 1-5 mM acetaldehyde activate the expression and activity of oncogenic transcription factor Jun/AP-1 in oral keratinocytes, thus acting as a tumour-promoting agent (Timmons et al., 2002). Furthermore, acetaldehyde treatment of human cells yields specific tandem base (GG to TT) substitutions in DNA. These mutations have also been found in upper digestive tract cancers (Matsuda et al., 1998). The mutational spectrum induced by acetaldehyde *in vitro* appears to resemble that of the p53 gene of oesophageal tumours

(Noori and Hou, 2001). This comparison, however, does not take into account the possible exposure to other carcinogens. In addition to the initiation of carcinogenesis, acetaldehyde may intensify the tumour development *in vitro* by increasing the proliferation rate of Caco-2 cells, reducing their adhesion and disturbing their differentiation (Koivisto and Salaspuro, 1998).

Very recently, a novel mutagenic mechanism induced by acetaldehyde has been suggested. With biologically relevant acetaldehyde concentrations (from 50-100 µM), polyamines were found to facilitate the formation of α-methyl-γ-hydroxy-1,n-propano-2-deoxyguanosine adducts, which previously have been detected only at supraphysiological (40 mM) concentrations of acetaldehyde. Furthermore, the polyamine spermidine reacted directly with acetaldehyde, generating crotonaldehyde (Theruvathu et al., 2005). Thus, in addition to direct adduct formation, acetaldehyde can be converted to crotonaldehyde by polyamines, forming crotonaldehyde adducts, which might contribute to the carcinogenicity of acetaldehyde *in vivo* in the digestive tract.

3.2.2 Acetaldehyde-related carcinogenicity in experimental animals

The carcinogenicity of acetaldehyde has been tested in several animal exposure experiments. In the IARC monograph, there is an extensive review of the topic. Acetaldehyde produces tumours of the respiratory tract by inhalation exposure. An increased incidence of laryngeal carcinomas in hamsters and adeno and squamous cell carcinomas of the nasal mucosa in rats are induced by inhalation exposure to acetaldehyde. However, another inhalation study with a lower acetaldehyde concentration and a study with intratracheal installation of acetaldehyde did not report increased tumour incidence. In these studies, acetaldehyde concentrations varied from approximately 30 µM to 130 µM and exposure times were 6-7 hours per day on 5-7 days per week. In hamsters, inhalation of acetaldehyde enhanced the incidence of respiratory tract tumours produced by benzo(a)pyrene (Feron et al., 1991; IARC, 1985; IARC, 1999; Woutersen et al., 1986). In the latest long-term experimental study in which acetaldehyde was given to rats for 161 weeks in tap water *ad libitum* in concentrations approximately from 1 mM to 56 mM, the total number of malignant tumours increased in all but one group. In particular, an increased incidence of carcinomas of nasal sinuses and oral cavity was detected in the groups treated with the highest dose (Soffritti et al., 2002). In an earlier study, hyperplastic and hyperproliferating changes in the tongue, epiglottis and forestomach were detected in rats receiving acetaldehyde (120 mM concentration) in tap water for eight months (Homann et al., 1997).

In conclusion, these studies strongly support the statement of the IARC that sufficient evidence exists in experimental animals to establish the carcinogenicity of acetaldehyde (IARC, 2004).

3.2.3 Acetaldehyde-related carcinogenicity in humans

Human exposure data with acetaldehyde is naturally scarce, since it would be unethical to conduct an exposure study – even for a short period – with a carcinogenic substance. However, an interesting finding was made in a survey at a chemical plant where workers were exposed to abnormally high concentrations of aldehydes such as acetaldehyde. Nine cancer cases were found among these workers. Of the cancer cases, five were bronchial tumours and two oral cavity carsinomas (IARC, 1985). Despite deficiencies in the study setting (poorly defined exposures, small number of cases), this finding supports the carcinogenic actions of acetaldehyde in humans.

As mentioned above, no proper human experimental studies exist on this topic. However, the natural genetic polymorphism of alcohol-, and acetaldehyde-metabolizing genes offers a human model for the carcinogenic effects of increased acetaldehyde exposure. As stated in the sections 1.3 and 2.2 and to be further discussed in the section 4.2, recent epidemiological and biochemical studies on ALDH2-deficient Asian and Caucasian alcoholics with high active ADH strongly support the role of acetaldehyde in alcohol-related digestive tract cancers.

In conclusion, strong evidence has been provided by experimental studies and human genetic models with different acetaldehyde exposures that acetaldehyde is a carcinogenic substance. Data on acetaldehyde-related carcinogenicity is summarized in Table 1.

Table 1.
Summary of acetaldehyde-related carcinogenicity *in vitro* and/or *in vivo*
(Only key references presented in parenthesis)

Experiment/test system	**Result**
IN VITRO	
Bacterial cell cultures	- Gene mutation - Aneuploidy (IARC, 1999)
Human/animal cell cultures	- Mutations/tandem base substitutions - Sister chromatid exchange - DNA-protein cross links - Chromosomal aberrations - Protein adducts - DNA strand breaks - DNA adducts - Activation of oncogenes - Interaction of polyamines, facilitating adduct formation (IARC, 1999; Theruvathu et al., 2005)
IN VIVO	
Cell damage *in vivo*	- DNA adducts in leukocytes of alcoholics (Fang and Vaca, 1997)
Inhalation exposure in animals	- Laryngeal carcinomas in hamsters - Nasal-mucosal carcinomas in rats (Feron et al., 1982; Woutersen et al., 1986)
Oral (drinking water) exposure in animals	- Carcinomas of nasal sinuses and oral cavity in rats - Hyperplastic and hyperproliferating changes in tongue, epiglottis and forestomach (Homann et al., 1997a)
Human "knock-out" model for increased acetaldehyde exposure during alcohol ingestion of individuals with impaired acetaldehyde metabolism (ALDH2 deficiency)	- Markedly increased cancer risk in upper digestive tract compared with individuals with normal acetaldehyde metabolism (Yokoyama and Omori, 2005; Yokoyama et al., 1998)

3.3 Tobacco smoking and acetaldehyde

As reviewed in previous sections, acetaldehyde is the major carcinogenic agent associated with alcohol-related upper aerodigestive cancers. However, acetaldehyde is also a well-known constituent of tobacco smoke. Since alcohol consumption often goes hand in hand with tobacco smoking and both are major risk factors for upper digestive tract cancers, a systematic review from the MEDLINE database was executed in order to clarify the relationship between acetaldehyde and smoking. The search of

Tobacco smoke OR smoking AND Acetaldehyde [59]

resulted in 59 studies, 19 of which were included in this review. Inclusion criteria comprised tobacco-derived acetaldehyde formation, concentration and possible effects *in vitro* and *in vivo*. The IARC monograph from 2004 on tobacco smoke and involuntary smoking was also included, as it presents some data on acetaldehyde as a constituent of tobacco smoke. Studies covering strict analytical methods or elimination of acetaldehyde (discussed in section 4.4) were excluded.

An estimated 4800 compounds are present in cigarette smoke, and 3044 constituents have been isolated from tobacco (Hoffmann and Hoffmann, 1997; IARC, 2004). Cigarette smoke contains several aldehydes generated by the combustion of organic material. Formaldehyde and acetaldehyde are listed as carcinogens in the IARC monograph. Other toxic aldehydes such as acrolein and crotonaldehyde have also been found from the tobacco smoke (Hoffmann and Hoffmann, 2001; Smith et al., 2003).

According to the IARC, the number of identified carcinogens in tobacco smoke was 69 in the year 2000. Eleven of these are known carcinogens and seven probable carcinogens in humans. Forty-nine are animal carcinogens and possibly also carcinogenic to humans (IARC Classification) (IARC, 2004). The exact number of human carcinogens is, however, unknown, since tobacco smoke contains over 80 toxic substances that could potentially act as carcinogens (Smith et al., 2003). Acetaldehyde is included in carcinogenic substances in tobacco mainstream smoke (MS) (Fowles and Dybing, 2003; Hoffmann and Hoffmann, 2001; Hoffmann et al., 2001). These carcinogens include 10 polynuclear (also called polycyclic) aromatic hydrocarbons (PAHs), six heterocyclic hydrocarbons, four volatile hydrocarbons, three nitrohydrocarbons, four aromatic amines, eight N-heterocyclic amines, 10 N-nitrosamines, two aldehydes (formaldehyde and acetaldehyde) and 10 miscellaneous organic, nine inorganic and three phenolic compounds (IARC, 2004). The most abundant of these carcinogens is acetaldehyde. Its concentration in non-filter cigarettes ranges from 500µg to 1400 µg. The concentration of acetaldehyde in cigarette smoke is more than 1000 times greater than that of PAHs and tobacco-specific nitrosamines (Hoffmann and Hoffmann, 2001). Thus, the concentration of acetaldehyde in mainstream smoke is far greater than the concentrations of the other carcinogenic substances (Rustemeier et al., 2002). Although the absolute concentration of a toxic substance is not directly comparable with its toxicity in humans, the amount of acetaldehyde in a single cigarette is far greater than that measured in humans

during ethanol elimination (to be discussed further in later sections).

When hazard prioritization for reported chemical constituents of cigarette smoke using toxicological risk assessment was provided by Fowles et al., aldehydes and small organic compounds were described as the main contributors to the overall cancer risk index (calculated by multiplying yield levels with cancer potency factors) (Fowles and Dybing, 2003). Moreover, acetaldehyde was placed in the first toxicity category with fifteen other substances (Smith and Hansch, 2000). In conclusion, besides acetaldehyde, many other toxic and carcinogenic substances are present in the mainstream smoke of cigarettes. It is impossible to evaluate the contribution of a single substance to the total carcinogenic effect. However, reducing the toxic potency of cigarette smoke might be possible, if the most significant causative agents of cancer could be identified and reduced. This will be further discussed in the sections covering the regulation of local acetaldehyde concentration in saliva.

As acetaldehyde is primarily in the gas phase, it is retained in the aerodigestive tract of smokers. As early as 1968, it was reported that 60% of acetaldehyde delivered in tobacco smoke is retained in the mouth (Dalhamn et al., 1968a). Another study assessed the retention of acetaldehyde to be 99% during inhalation of cigarette smoke (Dalhamn et al., 1968b). Although there is marked retention of acetaldehyde during smoking, this is not reflected in an increase in systemic blood acetaldehyde of smokers. This is understandable since acetaldehyde is a reactive and soluble substance acting locally. Acetaldehyde derived from smoking has previously been measured from the breath and saliva. After smoking a cigarette, breath acetaldehyde concentration is increased sixfold and that of saliva twofold (Annovazzi et al., 2004; McLaughlin et al., 1990). In the airways, acetaldehyde when inhaled has been shown to induce histamine-related bronchoconstriction in humans (Myou et al., 1993; Sanchez-Toril et al., 2000).

Few studies have tested the toxic effects of the aldehyde mixture present in tobacco smoke in cell cultures. In *in vitro* experiments, acetaldehyde and acrolein inhibit human bronchial epithelial cell repair processes similarly, to whole-cigarette smoke condensate (Wang et al., 2001). However, two other studies with acetaldehyde failed to induce radical toxic effects on a human b-cell line or on mouse lymphocytes. On the other hand, mixtures of different aldehydes (acetaldehyde, acrolein, formaldehyde, propionaldehyde) were able to significantly inhibit cell proliferation (Poirier et al., 2002) and to induce direct DNA damage (Yang et al., 1999). Acrolein was found to be a more potent toxicant than formaldehyde and acetaldehyde to human bronchial fibroblasts after treatment with micromolar concentrations. Furthermore, these aldehydes markedly inhibited the DNA repair enzyme methyl-guanine-DNA methyltransferase, as pointed out previously with acetaldehyde (Krokan et al., 1985). In addition, acetaldehyde and acrolein were observed to have cytotoxic effects, with disturbed adhesion and viability of cultured human gingival fibroblasts (Poggi et al., 2002). There may, however, be some flaws in *in vitro* cell studies since they have failed to show toxic effects of other toxicants in

cigarette smoke, e.g. benzene and styrene (Poirier et al., 2002).

The toxicity of tobacco smoke related to acetaldehyde is also related to the combined actions of other harmful substances in the tobacco smoke. One of these is the interaction of acetaldehyde with PAH compounds by enhancing the formation of benzo[a]pyrene DNA adducts (Barnes et al., 2000). The interaction of formaldehyde and acetaldehyde also produces newly identified cyclic deoxyguanosine adducts and formaldehyde cross links (Cheng et al., 2003). Aldehyde-derived promutagenic adducts have been found in smokers *in vivo*. Acrolein- and crotonaldehyde-derived propanodeoxygaunosine adducts were significantly higher in the DNA of gingival tissue of smokers than non-smokers (Nath et al., 1998). Even though *in vivo* studies concerning possible acetaldehyde-induced adducts in smokers' tissues were not found, the presence of such adducts can be expected since adduct formation of acetaldehyde with DNA has clearly been shown *in vitro* and also found *in vivo* in alcoholics.

In conclusion, considering the high exposure of smokers to acetaldehyde, acetaldehyde is likely to be a substantial factor behind smoking-related carcinogenic and other toxic effects.

4 REGULATION OF LOCAL ACETALDEHYDE CONCENTRATION IN THE UPPER DIGESTIVE TRACT

This last section will systematically review the known factors or individual characteristics by which local acetaldehyde concentration is regulated in the upper digestive tract. By affecting levels of carcinogenic acetaldehyde, one might be able to influence the risk of upper digestive tract cancer.

4.1 Role of microbes and oral hygiene

Human oral flora is known to contain over 350 cultivatable species (with another 50% uncultivatable by current techniques), and the bacteria may grow as densely as in the colon. The composition of oral flora, however, varies from place to place in the mouth. Thus, tooth surfaces, the buccal mucosa, the surface of the tongue and the pharynx all have different and characteristic flora. Many bacteria of the oral flora are facultative or aerobic bacteria, thus being able to produce acetaldehyde in the oral cavity with their ADH enzyme (Bagg et al., 1999). Consequently, oral microbes may have an essential contribution to the local acetaldehyde production individually. On the other hand, poor dental health associated with bacterial overgrowth is a risk factor for cancer of the mouth in alcoholics (Maier et al., 1993). To elucidate the regulatory role of microbes and oral hygiene in local acetaldehyde levels in the upper digestive tract, a systematic review was executed as follows:

Mouth OR pharynx OR larynx OR oesophagus
AND
Microbes OR Bacteria OR Fungi
AND
Acetaldehyde [4]

As a result, four studies were found from the MEDLINE and one additional study from the PUBMED.
Homann et al. have intensively studied the contribution of oral bacteria and hygiene to the local acetaldehyde production. First of all, they demonstrated a marked acetaldehyde production in saliva after ingestion of a moderate amount of ethanol (Homann et al., 1997b). Furthermore, considerable interindividual variation in acetaldehyde production capacity was also detected. This acetaldehyde production was significantly reduced after a three-day use of an antiseptic mouthwash – chlorhexidine (Homann et al., 1997b). This is a strong indicator that acetaldehyde production is of microbial origin. *In vitro* acetaldehyde production was also shown to correlate with the ethanol concentrations, to be linear over time and not be saturated under physiological ethanol conditions. The microbial acetaldehyde production is also supported by Muto's results, in which a certain *Neisseria* was identified from the human oral flora to possess extremely high ADH activity. This bacterial strain was also able to produce clinically significant amounts of acetaldehyde and more than 100-fold higher acetaldehyde levels (23 µM/min/10^{11} CFU) than other strains. Furthermore, alcohol ingestion influences the bacterial composition of the oral microbiota, resulting in an increased proportion of non-pathogenic *Neisseria* (Muto et al., 2000). Yeasts may also significantly contribute to local acetaldehyde levels in the oral cavity. Some *C. albicans* strains, restricted from human saliva, have a marked capacity to produce acetaldehyde from ethanol *in vitro* (Tillonen et al., 1999a).

Some evidence exists that bacterial overgrowth in patients with poor oral hygiene influences local acetaldehyde production in the mouth. A recent study in which the role of dental status in microbial production of acetaldehyde was evaluated, showed that poor dental status leads to a twofold increase in *in vitro* salivary acetaldehyde production from ethanol (Homann et al., 2001). Furthermore, microbiological analysis of "high" and "low" acetaldehyde-producing salivas obtained from human volunteers demonstrated that several bacterial strains were associated with increased acetaldehyde levels. "High acetaldehyde producers" were for instance *Streptococcus salivarius*, alpha-hemolysing *Streptococci*, *Corynebacterium* ssp. and *Stomatococcus* (Homann et al., 2000). The last study included in the review dealt with the invention of an antimicrobial agent, chlorhexidine, formulated as a controlled-release chip and fixed with a dental device. It was hypothesized that this might be a rational strategy for reducing acetaldehyde production by oral microbiota (Rota and Poggi, 2003).

In conclusion, large interindividual variations are present in oral acetaldehyde levels after consumption of alcohol, and oral microbes have an essential role in acetaldehyde production in the oral cavity. In addition, evidence is available for the contribution of poor dental hygiene to acetaldehyde production in the mouth.

Whether a certain strain or the total number of bacteria is more important in the regulation of individual acetaldehyde levels in saliva remains unknown.

4.2 Role of genetic factors

As stated in previous sections, in addition to environmental factors, genetic differences in alcohol- and acetaldehyde-metabolizing enzymes may have an influence on acetaldehyde production in the oral cavity. To further elucidate the role of genetic factors in the regulation of local acetaldehyde levels in the upper digestive tract, the literature was systematically reviewed as follows:

Mouth OR pharynx OR larynx OR oesophagus
AND
Alcohol dehydrogenase OR aldehyde dehydrogenase
AND
Acetaldehyde [3]

As a result, three studies were found (Muto et al., 2000; Seitz and Pöschl, 1997; Väkeväinen et al., 2000); the first was a review and the second was excluded since it investigated Neisseria's acetaldehyde production and was already discussed in the previous section. Due to the low number of MEDLINE results, the search was executed also in PUBMED. No studies concerning local acetaldehyde concentrations in the upper digestive tract in relation to ADH or ALDH were found. However, one study was found dealing with the expression of ALDH2 in the mucosa of cancer patients in relation to alcohol consumption (Morita et al., 2005). This study did not report *in vivo* acetaldehyde levels but was included in the review, since the finding could be attributed to local acetaldehyde. Results were complemented with a manually selected study in which the association of ADH1C*1 allele and salivary acetaldehyde was determined (Visapää et al., 2004).

Individuals with a mutated ALDH2 allele have increased systemic acetaldehyde levels during ethanol metabolism (Luu et al., 1995; Meier-Tackmann et al., 1990; Peng et al., 2002). For ALDH2*2 heterozygotes, the ingestion of 0.4 g of ethanol per kg of body weight results in a mean blood acetaldehyde level of approximately 23 µM (Mizoi et al., 1994). This is a significant concentration compared with the negligible levels of blood acetaldehyde during ethanol metabolism in individuals with normal ALDH2 enzyme activity (Sarkola et al., 2002). However, Väkeväinen et al., showed that the local acetaldehyde concentration in saliva after a dose of ethanol (0.5 g/kg of body weight) is approximately ninefold higher than blood acetaldehyde levels in ALDH2-deficient heterozygotes. Furthermore, the salivary acetaldehyde concentration in these flushers was two to three times higher than in subjects without the mutated allele (Väkeväinen et al., 2000).

Salivary acetaldehyde concentration has been demonstrated to be significantly modulated by the ADH1C genotype. Subjects homozygous for the ADH1C*1 allele have significantly higher salivary acetaldehyde concentrations following alcohol ingestion than non-homozygotes (Visapää et al., 2004). However, no differences in blood acetaldehyde levels were observed between individuals with different ADH genotypes. Furthermore, a

significant correlation between salivary acetaldehyde and salivary ethanol levels was found in all subjects. Only subjects with the ADH1C*1,1 genotype produced significantly more salivary acetaldehyde than the other genotypes. It was also illustrated that even with high salivary ethanol concentrations (40-50 mM) the respective acetaldehyde concentration curve did not seem to plateau, suggesting that acetaldehyde formation was saturated.

Changes in salivary acetaldehyde in this case probably could not be explained by changes in oral mucosal ethanol metabolism since class I ADH is not expressed in the oral mucosa (Yin et al., 1997). However, acetaldehyde might be produced in the salivary glands, as shown in the case of ALDH2-deficient subjects (Väkeväinen et al., 2000). On the other hand, both inactive and active forms of ALDH2 are induced in the oesophagus by heavy drinking, suggesting the accumulation of acetaldehyde locally (Morita et al., 2005). Thus, this finding supports the substantial role of alcohol and acetaldehyde metabolism in the regulation of acetaldehyde levels in the upper digestive tract. However, the contribution of mucosal enzyme activity to local acetaldehyde levels remains to be elucidated.

To summarize, there is strong evidence that genetic differences in ethanol- and acetaldehyde-metabolizing enzymes influence local acetaldehyde concentrations in the upper digestive tract. Thus, increased acetaldehyde levels in the saliva of ALDH2-deficient and ADH1C*1-1 subjects could be a major contributor to the increased cancer risk associated with ethanol consumption in these individuals. This concept is supported by epidemiological studies (section 1.3) showing that cancer risk in these subjects is particularly increased in the upper digestive tract, but not for example in the liver.

4.3 Role of tobacco smoking

As mentioned earlier, acetaldehyde is one of the main substances in tobacco smoke. Thus, smokers are exposed to this carcinogenic agent during inhalation of tobacco smoke. To evaluate the exposure of the human upper digestive tract to tobacco smoke-derived acetaldehyde, a systematic review was executed from the MEDLINE database as follows:
*Gastrointestinal tract AND tobacco OR smoking AND acetaldehyde [8].

Eight studies were found. Four of these were *in vitro* studies investigating the actions of cigarette smoke on human fibroblasts, and thus, have been referred to already. As a result, four studies from the MEDLINE search and two manually selected studies (Annovazzi et al., 2004; Seeman et al., 2002) were included in the review.

Smoking has been demonstrated *in vitro* to significantly increase salivary acetaldehyde production from ethanol. Smoking showed a positive linear correlation with salivary acetaldehyde and a smoker with a daily consumption of ~20 cigarettes was estimated to have an increased *in vitro* salivary acetaldehyde production of ~50-60% (Homann et al., 2000). In that study, the additional acetaldehyde was produced by the oral microbiota, and qualitative changes described in the oral flora of smokers were suggested to contribute to the increased acetaldehyde production.

Earlier *in vivo* evidence has indicated that acetaldehyde is markedly retained in smokers during cigarette smoking (Dalhamn et al., 1968a; Dalhamn et al., 1968b). However, systemic acetaldehyde levels are not elevated. Acetaldehyde levels in the breath have been shown to be significantly increased immediately after smoking (McLaughlin et al., 1990).

The retention of acetaldehyde in the upper digestive tract is supported by the only study found (in the review search) measuring aldehyde concentrations after smoking *in vivo* (Annovazzi et al., 2004). Acetaldehyde, formaldehyde and acrolein were measured from the saliva of smokers and non-smokers and from non-smokers after smoking one cigarette. The salivary aldehyde concentration of regular smokers was twofold higher than that of non-smokers. Moreover, the acute smoking of a cigarette increased the aldehyde concentration of saliva to 3.5-fold. However, the enormous baseline variation in the salivary acetaldehyde levels (from 21 µM to 463 µM) in study subjects is noteworthy. This could at least in part be due to the uncontrolled acetaldehyde formation in the saliva, e.g. by oral microbiota. Thus, it would be logical to conclude that a significant amount of acetaldehyde from tobacco smoke is dissolved in the upper digestive tract during smoking.

4.4 Role of cysteine as an acetaldehyde-binding agent

A short introduction to cysteine is given before the systematic review.

Cysteine is a sulphur-containing amino acid. With respect to human physiology, it is considered semiessential since it can be synthesized from methionine and serine via transsulphuration. Cysteine is normally consumed as a component of dietary proteins. The estimated average intake of cysteine is 1.3 g/day and 0.8 g/day in men and women, respectively. Dietary cysteine is absorbed as cysteine, cystine (two cysteine molecules attached by disulphide linkage) and cysteine-containing peptides. A substantial proportion of ingested free, sulphur containing amino acids are removed from portal circulation by the liver. *In vivo*, cystine − the oxidized form of cysteine is a more stable compound than cysteine; the respective plasma concentrations in humans are 40 µM and 8 µM.

The importance of cysteine is related to the presence of a sulphur-containing thiol group in its side chain (a thiol is a compound that contains the functional group composed of a sulphur atom and a hydrogen atom). Thus, cysteine plays a key role in the regulation of cellular redox status. Cysteine is also the rate-limiting amino acid in glutathione synthesis, which is a major cellular defence factor against reactive molecules in the body (Shoveller et al., 2005).

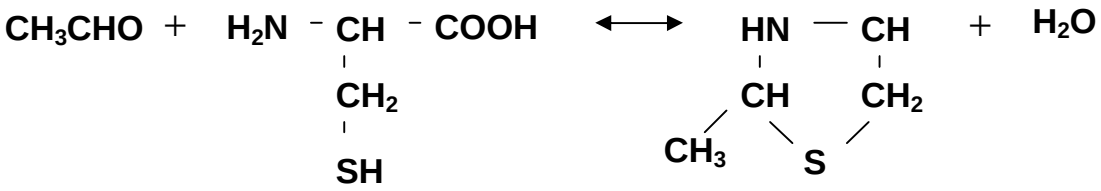
4.4.1 Interaction of acetaldehyde and cysteine

In this section, a review of the interaction of acetaldehyde and cysteine is given. The MEDLINE search was executed as follows:

Cysteine AND acetaldehyde [34].

From the resulting 35 studies, 14 were included, as they covered the interaction of acetaldehyde and cysteine *in vitro* or *in vivo*. Eight of these were experimental animal studies, five *in vitro* studies and one a human study. In the human study, plasma free glutathione and cysteine levels were measured after ethanol administration. In addition, two studies were manually selected (Susilo et al., 1989; Wlodek et al., 1993). None of the studies dealt with local acetaldehyde reduction or binding of acetaldehyde with cysteine in the human gastrointestinal tract. Studies dealing with systemic augmentation of glutathione with cysteine, peptide-bound cysteine or the effect of cysteine on ethanol-metabolizing enzymes were excluded.

For many decades, thiols have been known to be effective protective agents against certain harmful substances. A "Thiol defence" hypothesis was presented to explain the removal of free cysteine from the bronchial cells by the inhalation of compounds in cigarette smoke. Acetaldehyde was stated to be the main component responsible for thiol removal. Consequently, acetaldehyde was able to react with cysteine according to the following equation:



acetaldehyde + cysteine ↔ 2-methyl-thiazolidine-4-carboxylic acid (MTCA) + water

Experimental studies from the 1960s already showed a significant reduction of tobacco-smoke derived acetaldehyde in water solution supplemented with cysteine (Braven et al., 1967; Fenner and Braven, 1968). Many studies have since confirmed the ability of L-cysteine to form an adduct with acetaldehyde by the above-mentioned non-enzymatic condensation reaction at a physiological pH and temperature, subsequently forming 2-methyl-thiazolidine-carboxylic acid (MTCA) (Macdonald et al., 1977; Susilo et al., 1989; Ungar et al., 1973; Wlodek et al., 1993). *In vitro*, increasing amounts of cysteine progressively decreased the detectable content of acetaldehyde. Moreover, there was no release of acetaldehyde, i.e. the reaction was not reversible in a 20 min incubation (Cederbaum and Rubin, 1976b). Based on this reaction, cysteine was found to protect the mitochondria from acetaldehyde-induced injury *in vitro* (Cederbaum and Rubin, 1976a). Experimental studies in animals have shown the protective effect of L-cysteine against acetaldehyde also *in vivo* (O´Neill and Rahwan, 1976). High oral dose of L-cysteine (2 mM/kg) conferred an

excellent protection (80% survivors) against oral intubation of LD_{90} dose (18 mM/kg and 0.2 mM/kg) of acetaldehyde or acrolein, respectively. However, the best protection and 100% survival was offered by a mixture of L-cysteine, thiamin and l-ascorbic acid (Sprince et al., 1975; Sprince, 1985). Intraperitoneal injection of L-cysteine (300 mg/kg) resulted in faster ethanol oxidation and reduced blood, brain and liver acetaldehyde concentrations during ethanol metabolism in mice (Tsukamoto et al., 1990). The reduction of liver acetaldehyde levels by L-cysteine was confirmed by a study in rats (Ryle et al., 1987). Acetaldehyde-binding capacity of L-cysteine has also been shown in a human blood medium, in which acetaldehyde was reduced to 47% by L-cysteine *in vitro*.
Interactions of acetaldehyde and sulphydryls have been elucidated in only one human study. Plasma free glutathione and cysteine levels were measured following administration of 0.2 g/kg of body weight of ethanol in healthy volunteers and in alcoholics on disulfiram. In patients on disulfiram, but not in healthy controls, plasma cysteine decreased significantly, which was correlated with the rise in acetaldehyde, suggesting the binding of L-cysteine to acetaldehyde (Burgunder et al., 1988).

4.4.2 Problems related to the *in vivo* use of cysteine

Many researchers have examined the antioxidant effect of parenteral L-cysteine supplementation. Most of these studies have failed due to certain problems in the administration of cysteine. These studies were not systematically reviewed since this thesis is based on the local binding of acetaldehyde in the saliva by administrating a small dose of L-cysteine perorally. However, some studies will be discussed in the next paragraph to elucidate the differences between systemic and locally administered L-cysteine. The stability and metabolism of the condensation product of L-cysteine and acetaldehyde − thiazolidine-carboxylic acid − will also be discussed.

The administration of free cysteine has been suggested to be toxic. Oral, subcutaneous or intraperitoneal administration of cysteine has been reported to induce neurological damage in experimental animals. An excess of L-cysteine may act as a neuronal excitotoxin in sites that lack the blood-brain barrier or in developing animals with immature blood-brain barriers (Janaky et al., 2000). These effects have come about with doses of 1-2 g/kg of body weight of L-cysteine (Meister, 1989). In other words, the respective harmful dose in a 70 kg human would be 80 g, which is several 100-fold higher than the maximum dose described in our studies. In humans, cysteine-supplemented diets (6.5 mg/ kg/day) have been used without investigating of possible toxicity (Raguso et al., 2000).

The second problem with systemic administration of cysteine is the reactivity of this amino acid. In other words, it is unlikely that free, effective cysteine will reach the desired site of action through systemic delivery. Studies have in fact shown that systemic administration of cysteine itself is ineffective (Anderson and Meister, 1987). Thus, different kinds of cysteine prodrugs have been developed to improve delivery (Gwilt et al., 1998). However, these drugs are not likely to be effective in binding

acetaldehyde locally since they need metabolic alteration to be as active as cysteine.

As mentioned earlier, MTCA is formed from the reaction of acetaldehyde with cysteine. The formation and actions of MTCA *in vivo* are largely unknown. However, MTCA has been proposed to be also endogenously formed in the human body when L-cysteine inactivates endogenous or exogenous acetaldehyde. The further metabolism of the formed MTCA in humans is unclear. Some *in vitro* studies have shown that MTCA is decomposed in hours, yielding 50-55% of the original concentration of acetaldehyde (Speisky et al., 1985). It has also been suggested that methyl-djenkolic acid (MDA) is formed from the ring opening of MTCA. MDA was demonstrated in hydrolysates of mammalian tissue (Wlodek et al., 1993). The most probable explanation *in vivo* is endogenous nitrosation of MTCA, forming N-nitrosothiazolidine 4-carboxylic acid (NMTCA). Endogenous nitrosation of MTCA to NMTCA may have prevented detection of MTCA in the urine of rats simultaneously given cysteine and ethanol, or synthetic MTCA (Kallama and Hemminki, 1983; Nagasawa et al., 1975). NMTCA is easily formed *in vitro* when L-cysteine, acetaldehyde and nitrite are incubated together. When NMTCA is administered to rats, more than 90% of this compound is recovered unchanged from the urine and faeces. Furthermore, NMTCA is also detected in the urine of human volunteers without exogenous administration. Cigarette smokers have been shown to excrete higher levels of NMTCA than non-smokers. The detected NMTCA is suggested to develop when acetaldehyde from cigarette smoke reacts with endogenous L-cysteine to form MTCA, which undergoes nitrosation and yields NMTCA (Ohshima and Bartsch, 1984). It is likely that the carcinogenic potential of NMTCA is very low, if present at all, since the compound is not metabolically converted in experimental animals. Furthermore, toxic or other adverse biological effects of MTCA and NMTCA have not been reported. However, further studies are warranted to elucidate the final biological significance of these compounds since the excretion of nitrosated thiazolidines in humans has been established (Ohshima and Bartsch, 1984).

In conclusion, there is solid evidence that cysteine is able to bind to acetaldehyde and thus reducing acetaldehyde *in vitro* and *in vivo*. Furthermore, data strongly suggest that cysteine should be administered locally in order to be effective in acetaldehyde elimination. The thiazolidine derivative formed during condensation of cysteine and acetaldehyde probably undergoes nitrosation *in vivo*, forming NMTCA. This substance is detected *in vivo* from human urine, especially from smokers, indicating endogenous deactivation of acetaldehyde by cysteine.

AIMS OF THE STUDY

Alcohol consumption and tobacco smoking are the primary risk factors for oral, pharyngeal, laryngeal and oesophageal cancers. Furthermore, their combined use has a synergistic effect on cancer risk. The mechanisms behind the development of cancer associated with alcohol and smoking remain obscure. Recent epidemiological findings provide strong evidence for the carcinogenicity of acetaldehyde in humans. Carcinogenic acetaldehyde is the first metabolic product of ethanol and also a major constituent of tobacco smoke. Thus, determining acetaldehyde production and exposure in the human upper digestive tract during alcohol consumption and smoking is important. As acetaldehyde is one of the primary carcinogens, a reduction in local acetaldehyde concentration might offer substantial benefits. The objective here was to investigate the use of L-cysteine in the elimination of acetaldehyde from the saliva in order to reduce the local exposure of the human upper digestive tract to this carcinogen.

Specific aims were as follows:

1) To elucidate acetaldehyde production and ethanol oxidation capability of human intestinal strains of *Escherichia coli* in the different atmospheric conditions prevailing in the digestive tract.

2) To examine the combined effect of alcohol consumption and tobacco smoking on *in vivo* acetaldehyde levels of saliva.

3) To investigate the ability of L-cysteine, which was slowly released from a specially designed drug formulation, to reduce salivary acetaldehyde levels after the ingestion of alcohol.

4) To examine the ability of a L-cysteine-containing lozenge to bind acetaldehyde from saliva during smoking.

MATERIALS AND METHODS

1 ACETALDEHYDE PRODUCTION AND ETHANOL OXIDATION BY *ESCHERICHIA COLI* (I)

Culture conditions and bacteria studied

Bacterial strains (Table 1), with "high" (IH 50546) and "low" (IH 50817) expression of ADH activity were grown on Brucella agar plates supplemented with 5% defibrinated sheep blood in aerobic (i.e. air), microaerobic (6%, O_2) and anaerobic conditions at 35°C for 48 h.

Table 1. Bacteria investigated in Study I.

			ADH	
Name	Strain	Isolated from	Activity (nmol/min /mg)	K_m (mM)
Escherichia coli	IH 50546	Stool	354±28	2.78±0.8
	IH 50817	Stool	7.5±3.7	NM

NM= not measurable

ADH determinations

Bacterial cells were harvested from the plates after 8, 12, 22 and 48 hours of incubation to obtain the bacterial mass. For ADH determination 22-hours samples were used. The harvested cells were washed three times in 100 mM potassium phosphate and adjusted to a turbidity of 4 on the McFarland scale. An aliquot of this suspension was then sonicated in an ice path. To obtain the cytosol, the sonicate was centrifuged at 100 000g at 5°C for 60 min. Cytosolic ADH activity was then determined spectrophotometrically by measuring, after addition of ethanol (final concentration 25 mM), the reduction of NAD at 25°C in 100 mM glycine buffer (pH 9.6). Cytosolic protein concentration was determined by the Bio-Rad method (Bio-Rad protein assay; Hercules, Ca, USA), and the ADH activity was calculated as nanomoles of reduced NADH produced by 1 mg protein/min.

Determination of ethanol consumption and acetaldehyde production

The bacteria were grown on agar plates with 0 mM (control), and 51±2 mM (aerobic) or 59±1 mM (microaerobic) (ethanol plates) ethanol concentrations. Samples of culture media as agar blocks were excised with a scalpel and collected in a headspace sample vial after 0, 8, 12, 22 and 48 h of incubation. To test the volatile losses of acetaldehyde, some agar plates were sealed with parafilm, and no significant losses were detected. To avoid artifactual acetaldehyde formation, the vials were immediately closed and kept frozen at -20°C until analysed. Ethanol and acetaldehyde concentrations of the samples

were analyzed by headspace gas chromatography as described in section 1.5.

The ethanol consumption rate from the agar plate was obtained by dividing the initial supplemented dose by the estimated time to reach a zero concentration of ethanol in the agar. Non-inoculated agar plates, incubated in parallel with the test plates, were used as controls, and the values thus obtained were subtracted from the ethanol levels of the samples inoculated with bacteria. The non-inoculated agar plates were also used for the detection of artifactual acetaldehyde. Anaerobic and microaerobic conditions were achieved by using the anoxomat system (Mart, Lihtenvoore, The Netherlands), to evacuate and replace the normal atmospheric air with an anaerobic gas mixture (80% N_2, 10% H_2, and 10% CO_2). The microarobic atmosphere contained 6% O_2. The results of the ethanol consumption, fermentation and acetaldehyde production are based on two or more separate experiments.

2 EFFECT OF ALCOHOL DRINKING AND SMOKING ON SALIVARY ACETALDEHYDE (II)

Subjects and study design

Thirteen healthy volunteers (10 males, 3 females) took part in the study. Six of the volunteers were non-smokers, mean age 28.8±1 (range 26-35) years and seven regular cigarette smokers, mean age 39.5±3 (range 26-61) years. Information about the smoking status, alcohol consumption and nutritional habits was obtained by self-reported questionnaire. All volunteers were moderate alcohol consumers, with a weekly average consumption of 70 g or less of alcohol, and all were devoid of any clinical signs of poor oral hygiene. The average cigarette consumption among smokers was 18.5 ± 2.1 (range 15-30), and all had a smoking history of more than ten years. All of the volunteers were on normal Western diet, and none were vegetarians. The volunteers were divided into two separate groups: smokers and non-smokers. Two repeated measurements (study dates separated by at least a three-day interval) were done with the smokers, and the non-smokers were used as controls. Exclusion criteria were as follows: treatment with antibiotics or oral antiseptic in the past month, recent food or fluid intake and smoking or tooth brushing during the previous 30 min. All participants were requested to refrain from alcohol use for at least 24 h before the study.

Determination of the effect of ethanol ingestion on in vivo salivary acetaldehyde in smokers and non-smokers

To measure the salivary acetaldehyde derived solely from ethanol, both parallel groups (smokers and non-smokers) ingested 0.8 g ethanol/kg body weight in a standardized 10% v/v solution of absolute ethanol in distilled water/orange juice (50%/50%) within 30 min after baseline saliva collection. To remove local ethanol,

the subjects rinsed their mouths three times with water, and saliva samples were then taken every 20 min for 160 min.

Determination of the effect of combined ethanol ingestion and tobacco smoking on in vivo salivary acetaldehyde in smokers

To measure the salivary acetaldehyde derived from the combined and simultaneous use of ethanol and tobacco, the above-mentioned procedure was repeated in smokers. Smokers then smoked one cigarette every 20 min, and saliva samples were collected every 10 min for 160 min.

Determination of the effect of smoking on in vivo salivary acetaldehyde in smokers

To measure the salivary acetaldehyde derived solely from smoking, the smokers smoked one cigarette (without ethanol ingestion) within 5 min after measuring the base level of salivary acetaldehyde. Salivary samples were then collected every 5 min for 15 min.

Collection and analysis of salivary acetaldehyde

To obtain saliva samples, volunteers were told not to swallow the secreted saliva but to retain it in their mouth. After 5 min, the saliva was collected into a sample tube and 450 µl of saliva was immediately transferred into a vial containing 50 µl of PCA. Acetaldehyde and ethanol levels were analysed by headspace gas chromatography as described in section 1.5. Each *in vivo* measurement was done in duplicate.

3 BINDING OF SALIVARY ACETALDEHYDE BY L-CYSTEINE DURING ALCOHOL METABOLISM (III)

Subjects

Nine healthy male volunteers, mean age (±SEM) 27 ± 1 years, took part in the study. All of them were moderate alcohol consumers, with a weekly average consumption of 70 g or less of ethanol, and all were devoid of any clinical signs of poor oral hygiene. One of the subjects was a light smoker (less than 7 cigarettes per day). Exclusion criteria were as follows: treatment with antibiotics or oral antiseptic in the past month, recent food or fluid intake and smoking or tooth brushing during the previous 90 min. All participants were told to refrain ingesting ethanol for at least 36 hours before the study.

Study design

A paired, placebo-controlled design in which each subject served as his own control was used. The two study days were separated by at least 48 h. A commercially available paraffin wax chewing gum (Orion Diagnostics, Espoo, Finland) was used to stimulate the production of saliva, and the

volunteers were instructed to chew at all sites of the jaw to yield a representative sample. After baseline saliva collection (2 ml within 2 minutes), each volunteer fastened a placebo- or L-cysteine-containing tablet under their upper lip at the beginning of each measurement and the baseline saliva collection was repeated. After the second baseline saliva collection, each volunteer ingested 0.8 g of ethanol/kg body weight in a standardized 10% v/v solution of absolute ethanol in distilled water within 20 min. To remove local ethanol, the subjects rinsed their mouths with water, and saliva samples were then collected at 20- min intervals for 320 min.

L-cysteine-/ placebo drug formulation

The placebo tablet was composed of 130 mg of hydroxypropyl methylcellulose (HPMC) (Methocel K4M Premium EP, The Dow Chemical Company, USA), 6.9 mg of carbomer (Carbopol 971P NF, BF Goodrich Company, USA) and 1.4 mg of magnesium stearate (Ph.Eur.). The composition of the L-cysteine tablet was exactly the same, except that 130 mg of HPMC was reduced to 30 mg and replaced with 100 mg of L-cysteine (Sigma Chemical Co., USA). HPMC and carbomer enables the binding of the tablet to the gingiva and the slow release of L-cysteine.

In vivo salivary acetaldehyde production

To measure *in vivo* salivary acetaldehyde levels, 450 µl of saliva was immediately transferred into a vial that contained 50 µl of 6 mol/l PCA. Acetaldehyde and ethanol levels were analysed by headspace gas chromatography as described in paragraph 1.5. Each measurement was done in duplicate. In order to minimise the effects of artifactual acetaldehyde formation the baseline values were subtracted from the acetaldehyde levels measured during the experiment.

4 BINDING OF SALIVARY ACETALDEHYDE BY L-CYSTEINE DURING SMOKING (IV)

Subjects

Seven healthy volunteers (5 males, 2 females), mean age 28±1.9 (range 21-37) years, took part in the study. Information about the smoking status, alcohol consumption and possible medications was obtained by a self-administered questionnaire. Five of the volunteers were active smokers (more than 10 cigarettes/day, >5 years) and two were habitual smokers (less than 10 cigarettes/week). All of the volunteers were moderate alcohol consumers, with a weekly average consumption of 70 g or less, and all were devoid of any clinical signs of poor oral hygiene. Exclusion criteria were as follows: treatment with antibiotics in the past month, use of antiseptic mouthwash in the past week and smoking during the previous 60 min. Participants were told to refrain from alcohol consumption for at least 24 h before the study.

Study design

A placebo-controlled single-blinded study design, in which each subject served as his/her own control, was used. All volunteers smoked a total of five cigarettes (Marlboro, Philip Morris Finland Oy, Helsinki), with at least a 30 min wash-out period between, to test the placebo tablet and four tablets with different concentrations of L-cysteine. Before smoking, volunteers started to suck in randomized order, single-blinded tablets, containing 0 (placebo), 1.25, 2.5, 5 or 10 mg of L-cysteine. The tablets were designed to dissolve during cigarette smoking. In addition to L-cysteine (Fluka Biochemika, Bucks, Switzerland) the tablets contained 725 mg (in placebo tablet 745 mg) of Mannitole (Parteck M 200, Merck KgaA, Germany), 20 mg blackcurrant-flavour (Quest International, Naarden, the Netherlands) and 2% magnesium stearate (Ph.Eur., Merck, Darmstadt, Germany).

To measure *in vivo* levels of acetaldehyde during smoking, salivary samples were collected from each volunteer during smoking periods with each tablet: i) before smoking to measure the baseline (-5 to 0 min), ii) immediately after smoking (0-5 min collection), iii) 5 min after smoking (5-10 min collection) and iv) 10 min after smoking (10-15 min collection). To obtain the saliva samples, volunteers were told not to swallow the secreted saliva but to retain it in their mouths. After the 5-min collection period, saliva was spit into a sample tube, and 500 µl were transferred into a vial and analysed immediately. Acetaldehyde levels were analysed by headspace gas chromatography as described in section 1.5. Each *in vivo* measurement was done in duplicate.

To determine how rapidly the salivary acetaldehyde increases again after the tablet had dissolved, three volunteers smoked one cigar (Hofnar, Swedish Match Cigars B.V. Holland) in order to make the smoking time longer than 6.3 min (dissolving time of the tablet). Just before smoking, they started to suck tablet containing 5 mg of L-cysteine. Salivary samples were then taken at 3-min intervals for 20 min. The sample procedure was the same as described above.

5 MEASUREMENT OF ACETALDEHYDE AND ETHANOL BY GAS CHROMATOGRAPH

Acetaldehyde (Studies I-IV) and ethanol (Studies I- III) levels were analysed by using headspace gas chromatography (Perkin Elmer, Norwalk, CT, USA) in which the vials were heated to a temperature of 37°C, as reported earlier (Jokelainen et al., 1994). The conditions for the analysis were as follows: Column 60/80 Carbopack B/5% Carbowax 20 M, 2 m x 3mm, Supelco, Inc., Bellefonte, PA, USA); oven temperature, 85°C; transfer line and detector temperature 200°C; carrier gas flow rate (N_2) 20ml/min.

Artifactual formation of acetaldehyde from ethanol during protein precipitation was

measured as follows: PCA was added simultaneously with ethanol into additional vials which were not incubated (I, II). The acetaldehyde concentrations of these control samples were subtracted from the acetaldehyde values obtained from the samples after the incubation period. The interference of artifactual acetaldehyde formation in Study III was eliminated as the study subjects served as their own controls. In the study no IV, parallel salivary samples without the effect of smoking were used to control acetaldehyde formation *in vitro*. These values were subtracted from the *in vivo* concentrations.

6 STATISTICAL ANALYSIS

All values are expressed as means ± standard error of the mean (SEM) (I-III) or means ± SD (IV). The areas under the acetaldehyde concentration-time curves (AUC) were determined by using NCSS 2000 statistical software (239 North 1000 East, Kaysville, UT, USA) (II, III). Statistical differences between the study groups were analysed by Mann-Whitney rank-sum test (I, III, IV) or Wilcoxon signed-rank test (II). *P* values of less than 0.05 were regarded as significant.

7 ETHICAL CONSIDERATIONS

Study protocols dealing with patients or volunteers (II-IV) were approved by the Ethical Committee of the Helsinki University Central Hospital and by the Finnish National Agency for Medicines (II, IV). Informed consent was obtained from all subjects.

RESULTS

1 ADH-DEPENDENT ACETALDEHYDE FORMATION OF *ESCHERICHIA COLI* IN DIFFERENT OXYGEN TENSIONS (I)

ADH-mediated ethanol oxidation and acetaldehyde production were detected in both aerobic and microaerobic conditions *in vitro*. The human intestinal strain of *E. coli* (IH 50546), which possessed high ADH activity, was able to produce significant concentrations of acetaldehyde compared with the other *E. coli* strain with low ADH activity (IH 50817). Some ethanol and acetaldehyde was also produced by fermentation in anaerobic conditions.

In aerobic and microaerobic (6% of oxygen) conditions, the ethanol consumption rate of the high ADH activity possessing *E. coli* was markedly higher than that of the low ADH activity strain, as expected. The ethanol consumption rates in aerobic and microaerobic conditions are shown in Figure 1. The ethanol consumption rate of IH 50546 (high ADH activity strain) was associated with almost linear acetaldehyde production. The acetaldehyde levels produced in aerobic and microaerobic conditions reached several hundreds of µM concentrations *in vitro*. The acetaldehyde production of the two *E. coli* strains in aerobic conditions is presented in Figure 2.

Under anaerobic conditions, when oxygen was unavailable, ethanol was not consumed, as expected. However, both strains were capable of alcoholic fermentation, and up to 5 mM concentration of ethanol was produced. This was associated with acetaldehyde production of 73.7±14.5 µM with IH 50546 and 49.4±3.4 µM with IH 50817.

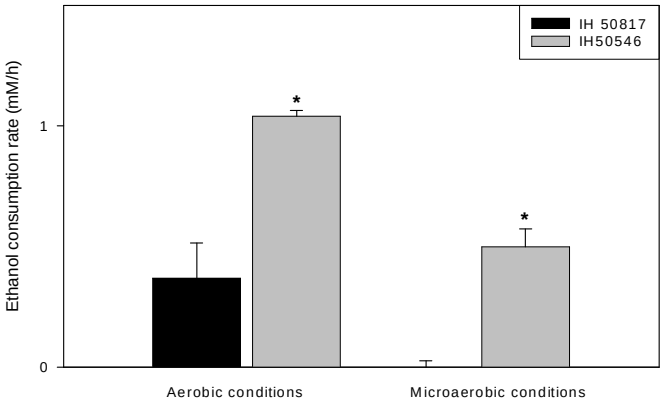


Fig. 1. Ethanol consumption rates (mean±SEM) of *Escherichia coli* IH 50817 and IH 50546 under aerobic and microaerobic conditions. * p<0.05. (reproduced with permission).

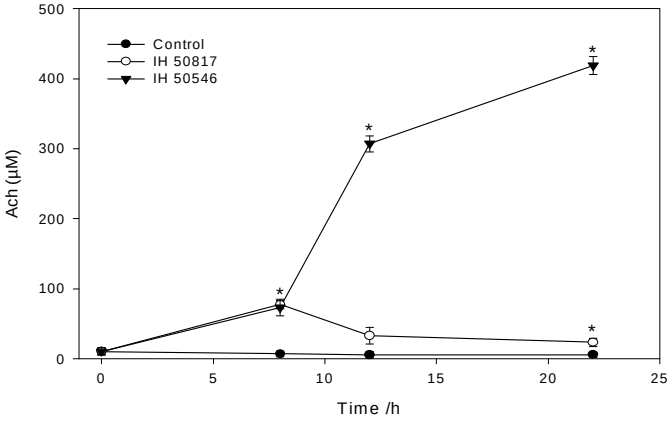


Fig. 2. Acetaldehyde concentrations (mean±SEM) in agar plates supplemented with ethanol and cultured under aerobic conditions with *Escherichia coli* IH 50817 or IH 50546. * p<0.05. (reproduced with permission).

2 SYNERGISTIC EFFECT OF ALCOHOL DRINKING AND SMOKING ON ACETALDEHYDE CONCENTRATION IN SALIVA (II)

Smokers without concomitant smoking during ethanol challenge had two times higher *in vivo* salivary acetaldehyde concentration than non-smokers. Moreover, with active smoking during the ethanol challenge, the salivary acetaldehyde exposure in smokers further increased, being sevenfold that of non-smokers.

The mean *in vivo* salivary acetaldehyde in smokers, even without smoking, after a moderate ethanol dose (0.8g/kg of body weight) was approximately two times higher than in non-smokers throughout the follow-up period of 160 min. The area under the curve (AUC) of salivary acetaldehyde in smokers and non-smokers was 114±12 and 54±9 µM x hr, respectively (p=0.002)(Fig. 3).

During active smoking, the *in vivo* salivary acetaldehyde was increased 10-fold over levels derived from ethanol ingestion alone in smokers. The AUC of salivary acetaldehyde in smokers with concomitant smoking and ethanol challenge was seven times higher than in non-smokers with ethanol challenge alone (370±12 and 54±9 µM x h, respectively (p<0.001) (Fig. 4).

The effect of smoking alone on *in vivo* salivary acetaldehyde level is shown in Figure 5. The acetaldehyde level is immediately increased to several hundred micromolars when smoking starts, declining rapidly after cessation of smoking.

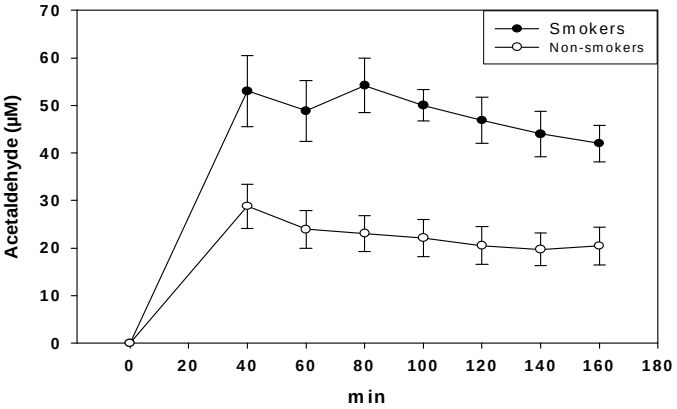


Fig. 3. In vivo salivary acetaldehyde levels (mean±SEM) after ethanol ingestion in smokers (without concomitant smoking) and in non-smokers. (Reproduced with permission).

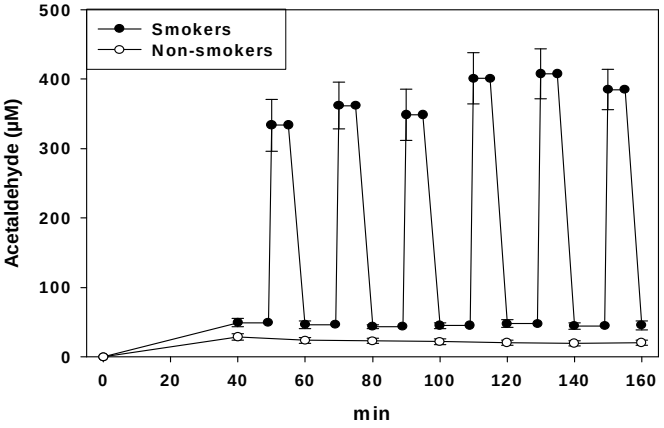


Fig. 4. *In vivo* salivary acetaldehyde after ethanol ingestion in smokers (with concomitant smoking) and in non-smokers. (reproduced with permission).

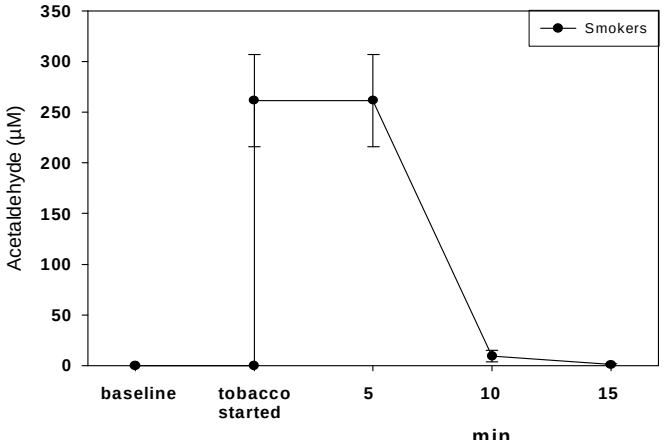


Fig 5. Salivary acetaldehyde in smokers after smoking one cigarette. (reproduced with permission).

3 ABILITY OF SLOW-RELEASING L-CYSTEINE TABLET TO BIND ACETALDEHYDE IN SALIVA DURING ALCOHOL METABOLISM (III)

The mean *in vivo* salivary acetaldehyde levels after ethanol ingestion were two to three times lower with the L-cysteine tablet than with the placebo. L-cysteine reduced the exposure of the oral cavity, pharynx and oesophagus to the carcinogenic acetaldehyde by 59 ± 8%. Thus, the L-cysteine tablet enables the binding of continuously formed acetaldehyde in saliva.

The AUC of salivary acetaldehyde with the L-cysteine tablet was 54 ± 11 µM x h and with the placebo 162 ±34 µM x h. The *in vivo* salivary acetaldehyde levels with the cysteine and placebo tablets are shown in Figure 6.

The corresponding salivary ethanol levels were equal in placebo and L-cysteine experiments throughout the follow-up.

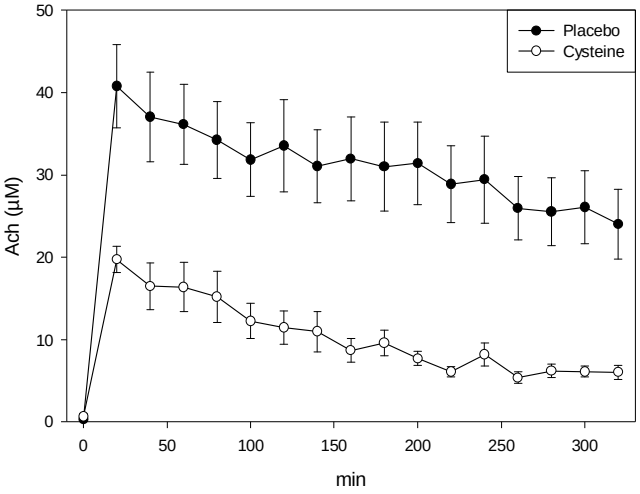


Fig. 6. *In vivo* acetaldehyde levels (mean±SEM) in saliva of volunteers with placebo or L-cysteine-containing buccal drug formulation after a dose of alcohol. Differences between concentrations are significant at all time points from 20 min to 320 min ($p \leq 0.001$). (reproduced with permission).

4 ELIMINATION OF ACETALDEHYDE FROM SALIVA BY L-CYSTEINE SUCKING TABLET DURING SMOKING (IV)

All of the tested tablets with different L-cysteine concentrations significantly reduced or totally eliminated the *in vivo* salivary acetaldehyde derived from smoking. However, the minimal L-cysteine concentration that was able to totally eliminate acetaldehyde from the saliva was 5 mg. Accordingly, the salivary acetaldehyde levels with placebo (0 mg) or 1.25, 2.5, 5, 10 mg of L-cysteine immediately after smoking were 228±15, 85±42, 9±7, 0.09±0.2 and 0±0 µM, respectively (Fig. 7).

The mean dissolving time of the lozenge was 6.3±0.8 min i.e. longer than one cigarette smoking period. After the tablet had dissolved, salivary acetaldehyde increased within 3 min to levels comparable with the placebo (Fig. 8).

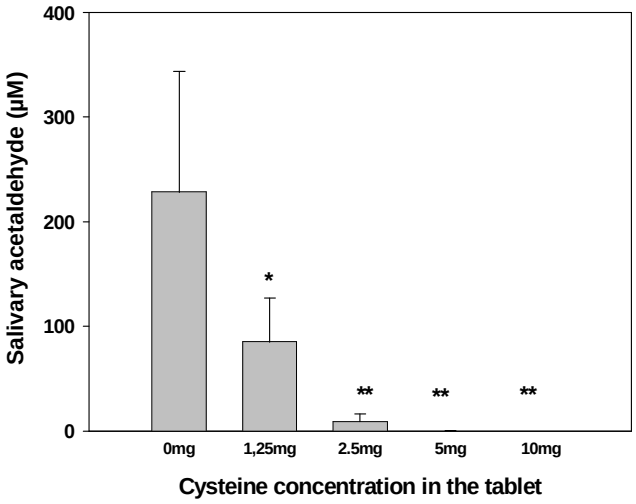


Fig. 7. Salivary acetaldehyde levels immediately after tobacco smoking with placebo- or L-cysteine-containing tablet. * p=0.007, ** p<0.001. (reproduced with permission).

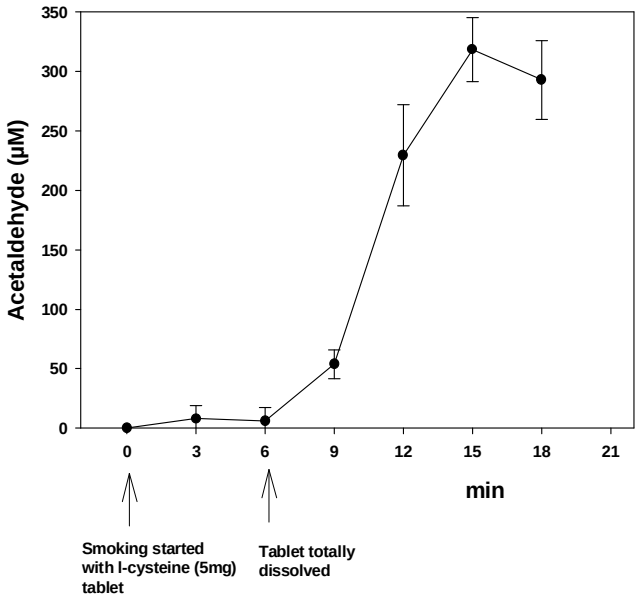


Fig. 8. Salivary acetaldehyde concentration *in vivo* during cigar smoking with a tablet containing 5 mg of L-cysteine. (reproduced with permission)

DISCUSSION

1 EXPERIMENTAL EVIDENCE OF MICROBIAL ACETALDEHYDE PRODUCTION UNDER DIFFERENT ATMOSPHERIC CONDITIONS PREVAILING IN THE DIGESTIVE TRACT

The gastrointestinal microbiota is exposed to significant ethanol concentrations during normal alcohol metabolism. Thus, microbially mediated ethanol oxidation, in which ethanol is oxidized to acetaldehyde, may lead to marked local acetaldehyde production in the digestive tract. Several studies have shown that human colonic contents are capable of oxidizing ethanol to acetaldehyde both *in vitro* and *in vivo*. This reaction is catalysed primarily by microbial alcohol dehydrogenase (ADH). Our results also show that ADH activity-expressing *E. coli* is capable of considerable ethanol oxidation and acetaldehyde production at ethanol concentrations in the gut comparable to those during normal drinking. Acetaldehyde production was strong in both aerobic and microaerobic conditions, but significant levels of endogenous acetaldehyde were also produced in anaerobic conditions.

The human microbiota consists of several hundred bacterial species and approximately 10^{14} individual bacteria. Thus, the microbiota can be considered a metabolically active organ. Bacterial count varies throughout the gastrointestinal tract, with for example local differences between the luminal and mucosal microbiota. In the colonic lumen, the oxygen tension is so low that for the most part only anaerobes can flourish. However, at the mucosal surface, oxygen diffusion from the mucosa is sufficient to maintain significant 0_2 pressure, capable of inhibiting the growth of strict anaerobes. Experimental studies have shown that pO_2 of intestinal mucosa is similar to that of venous blood. Accordingly, aerobes on the colonic mucosa are as numerous as or even more numerous than anaerobes. Thus, atmospheric conditions throughout the digestive tract may influence the composition and metabolism of the microbiota.

As presented in the results, under anaerobic conditions, both bacterial strains ("high" and "low" activity *E. coli*) carried out alcohol fermentation, producing ethanol via acetaldehyde, which both were produced at measurable levels. However, under aerobic and more importantly microaerobic conditions prevailing near the mucosa, only the high ADH activity *E. coli* were able to produce significant amounts of acetaldehyde. Thus, ADH-mediated ethanol oxidation is possible under different atmospheric conditions of the gut with varying oxygen tensions. Studies presented in this thesis were carried out with *E. coli*, but the results are probably also applicable to other microbes possessing ADH activity and to the entire digestive tract, from the oral cavity to the large intestine.

However, as it is known that aerobic and facultative anaerobic microbiota in the human digestive tract may contain various types of ADH enzymes with low or high activities and with different Km values,

differences may account for individual acetaldehyde levels in the saliva, gastric juice and the contents of the large intestine. Furthermore, since significant ethanol fermentation occurs under anaerobic conditions in different parts of the digestive tract, this endogenous ethanol can also be oxidized to acetaldehyde under the microaerobic conditions prevailing close to mucosal surfaces. Accordingly, significant levels of endogenous acetaldehyde are detected at least in the large intestine even without alcohol consumption (Salaspuro V et al., unpublished data).

In conclusion, our results suggest the existence of a complex and versatile microbial pathway for the production of carcinogenic acetaldehyde (Figure 9).

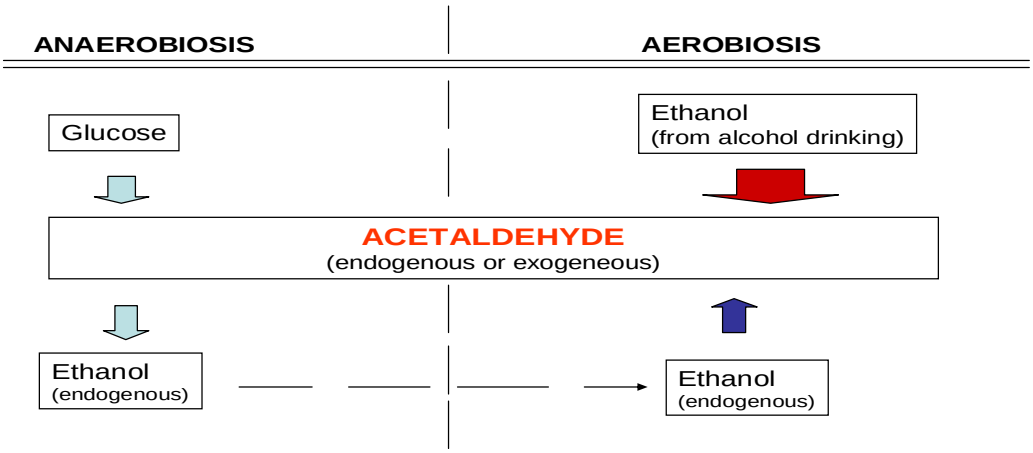


Fig. 9. Different pathways for microbial production of endogenous and exogenous acetaldehyde in the digestive tract from glucose or ingested ethanol under aerobic or/and anaerobic conditions.

2 MULTIPLICATIVE EXPOSURE TO CARCINOGENIC ACETALDEHYDE BEHIND THE SYNERGISTIC EFFECT OF ALCOHOL AND TOBACCO ON CANCER RISK

Tobacco smoking and alcohol consumption are the main risk factors for upper digestive tract cancers. When combined, alcohol and tobacco have a multiplicative effect on cancer risk, suggesting a synergistic tumour-promoting effect. The mechanism has thus far been poorly understood. However, as carcinogenic acetaldehyde is a major constituent of tobacco smoke that is dissolved in saliva and is also locally produced from ethanol in the upper digestive tract, excessive exposure to this carcinogenic substance might be a potential explanation of the markedly increased

cancer risk associated with combined smoking and drinking.

Smokers, even without acute smoking, were shown to have two times higher salivary acetaldehyde concentration than non-smokers during ethanol challenge. This implies that smokers have changes in their oral microbiota, resulting in an increased capacity to produce acetaldehyde from ethanol, as previously also shown *in vitro* (Homann et al., 2000). Moreover evidence exists of the inhibition of mucosal ALDH enzyme mediated by toxic tobacco smoke, which could result in less efficient acetaldehyde removal and higher acetaldehyde concentration in the aerodigestive tract of chronic smokers (Helander and Curvall, 1991).

Our second finding was that acetaldehyde is easily and rapidly dissolved from tobacco smoke into the saliva during smoking. Accordingly, smoking alone increases *in vivo* salivary acetaldehyde to over 250 µM during every smoking period. However, large interindividual variations were present in salivary acetaldehyde, ranging from 100 µM to 400 µM. The total exposure of smokers to acetaldehyde is large influenced by smoking frequency and whether or not tobacco smoke is inhaled. Nevertheless, these *in vivo* acetaldehyde levels have been shown to be mutagenic. Due to the deposition of acetaldehyde into the aerodigestive tract, the carcinogenic acetaldehyde also reaches the oesophagus and stomach via normal wash-out of saliva. This provides a sound explanation for the increased oesophageal and stomach cancers risks observed in smokers and alcohol drinkers.

This multifold increase in salivary acetaldehyde concentration may have a pronounced effect on the upper digestive tract cancer risk of heavy drinkers, who are often also heavy smokers. The combined use of alcohol and tobacco dramatically increased salivary acetaldehyde. Consequently, salivary acetaldehyde levels after an ethanol dose were seven times higher in smokers with active smoking (every 20 min) than in non-smokers. Although, salivary acetaldehyde declined rapidly, the basal level in smokers was still two times higher than in non-smoking controls during ethanol challenge.

In conclusion, continuous acetaldehyde exposure in smokers during alcohol consumption is double that of non-smokers. Furthermore, active smoking induces dramatic but momentary acetaldehyde peaks in saliva. With simultaneous smoking and drinking, there is a markedly increased exposure to carcinogenic acetaldehyde, which may explain the synergistic and multiplicative risk effect of alcohol and smoking on upper digestive tract carcinogenesis.

3 REDUCING EXPOSURE OF THE UPPER DIGESTIVE TRACT TO CARCINOGENIC ACETALDEHYDE BY L-CYSTEINE AFTER ALCOHOL CONSUMPTION

A plausible explanation for the increased upper digestive tract cancer risk in alcoholics and heavy alcohol consumers is the increased local acetaldehyde concentration in saliva, which, via swallowing, is distributed to all parts of the upper digestive tract. This theory is strongly supported by epidemiological studies on ALDH2-deficient Asian alcohol consumers, who possess abnormally high cancer risk associated with high local acetaldehyde levels in saliva after ingesting alcohol. The theory is also supported at least in part by studies on Caucasian alcoholics and heavy drinkers who are homozygous for the fast-alcohol metabolizing (ADH1C*1) enzyme, resulting in abnormally high salivary acetaldehyde concentration after alcohol consumption and also in an increased risk of alcohol-related upper digestive tract cancers.

In theory, the harmful effects of reactive acetaldehyde could be prevented by binding it to L-cysteine. Thiol compounds, such as cysteine, can bind aldehydes *in vitro* and protect against acetaldehyde toxicity *in vivo*. Cysteine is able to inactivate the reactivity of acetaldehyde by non-enzymatic binding, forming a more stable compound, 2-methylthiazolidine-4-carboxylic acid. Cysteine is a semi-essential amino acid that is present after protein digestion also in the normal diet. The protective role of L-cysteine in humans has thus far been thought to be linked to its systemic effects via intracellular glutathione, as cysteine is the rate-limiting amino acid in the synthesis of glutathione. The hypothesis presented here, however, is related to the local use of L-cysteine to bind acetaldehyde *in situ*, without its systemic delivery. This is associated with several benefits including, (i) cysteine would not have to be absorbed from the gut, (ii) formed acetaldehyde is directly and immediately bound at the site of formation and (iii) the total amount of delivered cysteine could be kept low.

To bind continuously formed acetaldehyde during ethanol oxidation in the oral cavity, a special buccal drug formulation was developed. This tablet slowly releases intact L-cysteine, which enables continuous and direct local binding of reactive acetaldehyde. Consequently, up to two-thirds of carcinogenic acetaldehyde can be removed from saliva after alcohol intake. This drug formulation could potentially be used to prevent the toxic effects of acetaldehyde during alcohol consumption. This could be of special benefit to individuals with increased acetaldehyde exposure during drinking, e.g. ALDH2-deficient Asians and subjects who are homozygous for the ADH1C1 enzyme. Since the L-cysteine tablet was found to be effective and safe in *in vivo* use, these findings warrant further clinical trials.

4 REDUCING EXPOSURE TO CARCINOGENIC ACETALDEHYDE BY L-CYSTEINE DURING SMOKING

As stated in the literature review, acetaldehyde is one of the major toxic components of tobacco smoke. Acetaldehyde is easily dissolved from the mainstream smoke into saliva and further to the entire aerodigestive tract of smokers. This could be one of the major mechanisms by which acetaldehyde, and probably also some other carcinogens of tobacco smoke might mediate toxic and carcinogenic effects. L-cysteine released slowly from the buccal tablet can effectively bind ethanol-derived salivary acetaldehyde. Moreover, orally administered L-cysteine can totally eliminate the tobacco smoke-derived acetaldehyde from saliva during smoking.

We have previously shown that smokers and heavy drinkers have increased *in vitro* production of acetaldehyde in saliva (Homann et al., 2000; Salaspuro, 2003). Here, we have further demonstrated that tobacco smoking dramatically increases salivary acetaldehyde concentration *in vivo*. These findings support our hypothesis on the carcinogenic role of local acetaldehyde in the upper digestive tract. Systemic acetaldehyde levels are negligible during both drinking and smoking.

All acetaldehyde was eliminated from the oral cavity during smoking with a rather small dose of L-cysteine (5 mg). As mentioned in the literature review, cysteine as a thiol compound forms a stable thiazolidine carboxylic acid (MTCA) when reacting with acetaldehyde. MTCA has also been shown to be endogenously formed in the human body when L-cysteine inactivates endogenous or exogenous acetaldehyde. Despite our limited understanding of MTCA, evidence indicates that MTCA undergoes endogenous nitrosation, forming nitrosothiazolidine 4-carboxylic acid (NMTCA) (Ohshima and Bartsch, 1984). This substance has been detected in the urine of human volunteers without exogenous administration of the precursors. Cigarette smokers have also been shown to excrete higher levels of NMTCA than non-smokers. The NMTCA has been suggested to develop when acetaldehyde from cigarette smoke reacts with endogenous L-cysteine (Ohshima and Bartsch, 1984). The L-cysteine lozenge offers a supplemental chemopreventive agent that binds acetaldehyde before it interacts with endogenous detoxification mechanisms.

The results of this thesis strongly suggest that a cysteine tablet sucked during every smoking episode could serve as a chemopreventive, acetaldehyde-eliminating agent. Moreover, its use can be considered safe; no reports have been made of harmful effects of L-cysteine (at the concentrations described here) or cysteine-acetaldehyde metabolites. However, even with the elimination of acetaldehyde, other important carcinogens remain in tobacco smoke. Smoking should therefore be avoided. Nevertheless, as approximately 43-63% of upper gastrointestinal tract cancers are attributable to tobacco smoking, even a small reduction in the carcinogenicity of cigarette smoke would provide considerable benefits. Further elucidation of the effects of the cysteine tablet on cancer prevention among smokers in larger clinical trials is warranted.

SUMMARY AND CONCLUSIONS

The main conclusions of this thesis are:

1. *In vitro* results with *E. coli* demonstrate that individual bacteria representing the normal microbiota of the human gastrointestinal tract can be a contributing factor in the previously described microbial pathway for ethanol metabolism. Aerobic and, more importantly, microaerobic conditions prevailing on the mucosal surface of the gut enable ADH-mediated ethanol oxidation. This may also lead to marked formation of carcinogenic acetaldehyde from ethanol *in vivo*. Furthermore, toxic acetaldehyde may be produced by bacteria without exogenously provided ethanol during anaerobic fermentation. This may contribute to the individual levels of endogenous acetaldehyde.

2. Smokers, as compared with non-smokers, have significantly increased acetaldehyde exposure during ethanol metabolism, even when not smoking. This most probably is due to changes in the oral microbiota of chronic smokers. Moreover, acetaldehyde exposure is dramatically increased during active tobacco smoking due to the significant deposition of smoke-derived acetaldehyde into saliva. Thus, the increased aerodigestive tract cancer risk observed in smokers may be the result of the acetaldehyde exposure from tobacco smoke. Furthermore, the markedly increased exposure to carcinogenic acetaldehyde caused by simultaneous smoking and drinking may explain the synergistic and multiplicative effect of alcohol consumption and smoking on upper digestive tract carcinogenesis.

3. Acetaldehyde produced in the oral cavity by microbial ethanol oxidation could be significantly decreased by a new buccal L-cysteine-releasing drug formulation. L-cysteine efficiently binds reactive acetaldehyde by forming a stable thiazolidine compound, thus preventing acetaldehyde from interacting with cellular proteins and DNA. As two-thirds of acetaldehyde can be trapped in saliva, our finding presents a means of decreasing the acetaldehyde exposure of individuals who have a greater cancer risk due to increased acetaldehyde concentration in saliva, such as ALDH2-deficient Asians, Caucasians with high activity ADH, smokers and heavy drinkers. Thus, buccal cysteine tablets could potentially be used to prevent upper digestive tract cancers induced by ethanol-derived acetaldehyde.

4. Study IV corroborates previous results that acetaldehyde – the major volatile substance of tobacco smoke is dissolved in saliva during smoking. This acetaldehyde can be completely removed by a L-cysteine lozenge. Hence, cysteine tablet sucked during every smoking period could be used to minimize acetaldehyde exposure, thus potentially preventing upper digestive tract cancers in smokers. However, it should be born in mind that smoking remains harmful because cysteine is not likely to inactivate all carcinogens present in tobacco smoke.

In conclusion, undisputed evidence reveals that alcohol consumption and tobacco smoking are the main risk factors for upper digestive tract cancers in humans. Strong

experimental and human genetic linkage data also indicate that acetaldehyde is one of the main contributors to the carcinogenic effect. This thesis confirms the earlier findings and further elucidates the role of acetaldehyde in the pathogenesis of alcohol- and smoking-induced cancers. We also present a novel experimental approach to decreasing local acetaldehyde exposure of the upper digestive tract with L-cysteine, with the eventual goal of reducing the prevalence of upper digestive tract cancers.

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